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RESPONSES OF TWO STRAINS OF RATS TO RAPESEED OIL AND CORN OIL[1]

JOYCE L. BEARE, T. K. MURRAY, AND J. A. CAMPBELL

Abstract

Wistar and Sprague–Dawley rats were compared in their response to 20% rapeseed oil or corn oil in a basal diet of ground fox cubes. Apparent coefficients of digestibility were significantly lower for rapeseed oil than for corn oil in the Sprague–Dawley, but not in the Wistar rats. After 6 weeks the adrenals of Sprague–Dawley rats were larger in the animals fed rapeseed oil than in those fed corn oil, while the adrenals of Wistar rats were not influenced by the dietary oil. These strain differences explain some discrepancies appearing in the literature concerning the effects of rapeseed oil in the rat.

In another experiment, Wistar and Sprague–Dawley rats deprived of vitamin A for 18 days were fed a purified basal diet with 20% rapeseed oil or corn oil and dosed with 75 or 150 I.U. of vitamin A per day. After 14 days there was no significant effect of the strain of rat or the type of dietary oil on the liver storage of vitamin A. The rate of depletion of vitamin A from the liver following a single dose of 1200 I.U. of vitamin A was also studied and indicated no significant difference attributable to the dietary oils.

Introduction

During investigation of rapeseed oil with inbred Wistar rats fed a purified basal diet, it was found that corn oil was more completely digested than rapeseed oil, although both oils had an apparent coefficient of digestibility greater than 90% (1). Deuel reported the digestibility of refined rapeseed oil to be 82% for rats fed a semipurified diet (2), and 99% for humans consuming a mixed diet low in fat (3). In contrast to these results, Carroll (4), working with Sprague–Dawley rats fed a basal diet of fox cubes, determined the coefficient of digestibility of rapeseed oil to be 58%. Results from different strains of rats also lacked agreement in the weight response of the adrenal glands to rapeseed oil (5). While vitamin A storage in the liver was found to be similar for inbred Wistar rats supplied with rapeseed oil or corn oil (1), Carroll (6) suggested that the requirement of Sprague–Dawley rats for vitamin A was increased by erucic acid, the predominant fatty acid of rapeseed oil. In the light of these divergent findings it was decided to investigate the response of two strains of rats, inbred Wistar and Sprague–Dawley, to dietary rapeseed oil and corn oil.

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Contribution from the Food and Drug Laboratories, Department of National Health and Welfare, Ottawa.

Procedures

Male rats, 3 to 4 weeks of age, of inbred Wistar and Sprague–Dawley strains, were randomized within 10 blocks according to initial body weight and litter. Each block consisted of four animals, one of each strain receiving ad libitum either 20% rapeseed oil[2] or corn oil in a basal diet of ground fox cubes.[3] Food consumptions and body weights were recorded twice weekly. During the second week all feces were collected for fat analyses by the procedures previously described (5). Apparent coefficients of digestibility for individual rats were calculated as the percentage of dietary fat not excreted in the feces. After 6 weeks on experiment, the animals were anesthetized with ether; blood was collected from a neck incision; and the liver, adrenals, and testes were removed and weighed. Cholesterol was determined by the method of Henly (7), and liver lipids by the method of Folch *et al.* (8).

To study the storage of vitamin A administered with rapeseed oil or corn oil, 50 male inbred Wistar and 50 male Sprague–Dawley rats were first fed a vitamin-A-free diet (9). After 18 days of this regimen, 10 animals of each strain were killed and the amount of vitamin A in the liver of each animal was determined by the method of Ames, Risley, and Harris (10). The remaining animals were fed purified diets containing 20% rapeseed oil or corn oil, similar to those previously described (1) except for the omission of vitamin A, and were dosed orally with either 75 or 150 I.U. of vitamin A in 0.1 ml of rapeseed oil or corn oil for 14 consecutive days. Two days later the animals were killed, and liver vitamin A determined.

The rate of depletion of vitamin A from the liver after administration of a single dose of the vitamin was studied with 40 weanling male rats of each strain maintained on the vitamin-A-free diet containing 20% rapeseed oil or corn oil. After 18 days of vitamin A deprivation, each rat received orally 1200 I.U. of vitamin A in 0.1 ml of the appropriate dietary oil. Forty-eight hours later six animals from each group, representing each strain and dietary oil, were killed, organs weighed, and the liver vitamin A determined. At intervals of 16 and 32 days following the single administration of vitamin A, seven animals of each group were likewise treated.

Results and Discussion

The mean weight gains of rats receiving 20% corn oil or rapeseed oil in a basal diet of ground fox cubes are shown in Table I. At the end of 1 week the Wistar rats receiving rapeseed oil exhibited weight gains which were 34.7% less than those of the Wistar rats fed corn oil while the comparable difference in the larger weight gains of Sprague–Dawley rats was 6.7%. The effect of rapeseed oil on the Sprague–Dawley rats increased in the succeeding weeks, but throughout the 6 weeks of the experiment the difference in weight gain response to the dietary oils was greater in the Wistar rats. After 4 weeks, the differences in weight gain were dependent on earlier growth rates and not on

[2]Golden rapeseed oil containing 37.0% erucic acid, supplied through the courtesy of Dr. B. M. Craig and the Saskatchewan Wheat Pool.

[3]Master fox cubes, obtained from Toronto Elevators, Ltd.

TABLE I

Mean weight gains of Wistar and Sprague–Dawley rats fed corn oil (CO) and rapeseed oil (RSO), and the percentage decrease in weight gain of those fed rapeseed oil compared to corn oil

	Weight gains, g					
	Wistar			Sprague–Dawley		
Weeks	CO	RSO	% decrease	CO	RSO	% decrease
1	14.2	9.3	34.7	35.5	30.3	6.7
2	38.5	21.6	43.9	62.0	47.9	22.7
4	84.9	51.9	39.9	120.3	95.1	20.9
6	132.3	96.1	27.4	171.6	144.0	14.5
4–6	47.4	45.1	4.9	51.3	48.9	4.8

the gains obtained from 4 to 6 weeks. The effect of rapeseed oil in decreasing weight gains was observed previously in Wistar rats (11, 5) and in Sprague–Dawley rats (12).

As shown in Table II the apparent coefficient of digestibility of corn oil was not significantly different in the two strains of rats, but the digestibility

TABLE II

The apparent coefficients of digestibility of corn oil and rapeseed oil fed to Wistar and Sprague–Dawley rats

Strain	Dietary oil	App. coeff. of dig.
W	CO	89.1±0.5*
W	RSO	83.0±1.5
S	CO	83.9±2.7
S	RSO	64.8±4.0

*Mean ± standard error.

of rapeseed oil was significantly lower in the Sprague–Dawley rats. This strain difference in the response to rapeseed oil appears to account for some discrepancies in the coefficients of digestibility appearing in the literature. In Table III are shown data on organ weights, liver lipid concentrations, and

TABLE III

Liver, testes, and adrenal weights, liver lipid concentrations, and adrenal cholesterol values of Wistar and Sprague–Dawley rats fed corn oil and rapeseed oil

Strain	Dietary oil	Liver weight, g	Testes weight, g/pair	Adrenal weight, mg/pair	Adrenal cholesterol, mg/pair	Liver lipids, %
W	CO	6.72±.43	2.54±.04	27.4±0.2	0.78±.07	6.97±.35
W	RSO	6.04±.53	1.69±.20	25.8±1.2	1.01±.06	7.31±.24
S	CO	9.76±.56	2.90±.23	45.1±2.2	1.65±.12	8.44±.28
S	RSO	10.06±.43	2.64±.21	64.2±3.7	2.41±.18	8.15±.39

adrenal cholesterol levels in these animals. Sprague–Dawley rats had larger livers than Wistar rats, but in neither strain was the liver weight significantly affected by the dietary oil. However, when liver weights were adjusted for body weights by a covariance analysis, those of rats fed rapeseed oil were

significantly heavier, as found previously (1), and the strain effect was negligible. The testes weights were similar, except in the group of Wistar rats fed rapeseed oil in which the testes were smaller. The adrenals of Sprague–Dawley rats were larger than those of Wistar rats, and were significantly increased in size by rapeseed oil, which had no comparable effect on Wistar rats. This strain difference in the response of the adrenal glands to rapeseed oil further accounts for lack of agreement between results from different laboratories. Significant differences in total adrenal cholesterol paralleled those of adrenal weight, and in the Sprague–Dawley rats corresponded to those previously reported by Carroll (12). The total lipid concentrations of the liver were unaffected by the dietary oils in the two strains.

In the second study, the livers of Wistar and Sprague–Dawley rats contained 11 and 88 units of vitamin A respectively prior to the daily administration of the vitamin. The appropriate basal value was subtracted from total vitamin A of the liver found after dosing. The results obtained from these animals are shown in Table IV. Wistar rats supplied with corn oil or rapeseed

TABLE IV

Organ weights, concentration of serum cholesterol, and liver vitamin A of Wistar and Sprague–Dawley rats deprived of vitamin A and then fed corn oil and rapeseed oil with two levels of vitamin A

Strain	Dietary oil	Vit. A dose, I.U./day	Body weight gain, g	Liver weight, g	Testes weight, g/pair	Adrenal weight, mg/pair	Serum cholesterol, mg/100 ml	Liver vit. A,* I.U.
W	CO	75	55 ± 2	5.03 ± .16	1.65 ± .09	21.7 ± .57	149 ± 14	400 ± 68
W	RSO	75	45 ± 4	5.44 ± .13	1.67 ± .07	23.1 ± .92	145 ± 12	463 ± 25
W	CO	150	59 ± 2	5.60 ± .14	1.74 ± .05	22.5 ± .45	139 ± 14	902 ± 31
W	RSO	150	50 ± 4	5.70 ± .27	1.63 ± .07	25.6 ± .66	153 ± 16	1079 ± 62
S	CO	75	89 ± 5	7.19 ± .33	2.21 ± .12	31.8 ± 1.27	142 ± 10	416 ± 32
S	RSO	75	79 ± 6	7.97 ± .56	1.86 ± .16	32.2 ± 1.23	130 ± 7	436 ± 40
S	CO	150	90 ± 5	7.76 ± .34	2.35 ± .07	30.0 ± .76	111 ± 13	954 ± 43
S	RSO	150	82 ± 6	8.38 ± .51	2.19 ± .25	30.5 ± .96	109 ± 12	937 ± 51

*Corrected for the initial amount of liver vitamin A in each strain of rats.

oil exhibited smaller body weight gains and smaller liver, testes, and adrenal weights than the corresponding Sprague–Dawley rats, and had similar values for serum cholesterol and liver vitamin A. The amount of vitamin A stored was approximately half of that administered in all cases. Under the conditions of this experiment no significant difference in vitamin A storage could be attributed to the type of dietary oil or the strain of rat. A repeat of this experiment confirmed these results.

The results obtained from the rats which received a single dose of vitamin A following 18 days of deprivation of the vitamin are shown in Fig. 1. Significant differences in body weight, food consumptions, and testes and adrenal weights were found 32 days after dosing. The liver weights and amounts of liver vitamin A were similar for animals fed the two dietary oils in both strains of rats. It appears, therefore, that differences in body weights, resulting from differences in dietary oils, do not influence the rate of depletion of vitamin A from the liver.

Carroll (6) reported that erucic acid, fed in a diet lacking in fat-soluble vitamins and essential fatty acids, increased the requirement for vitamin A,

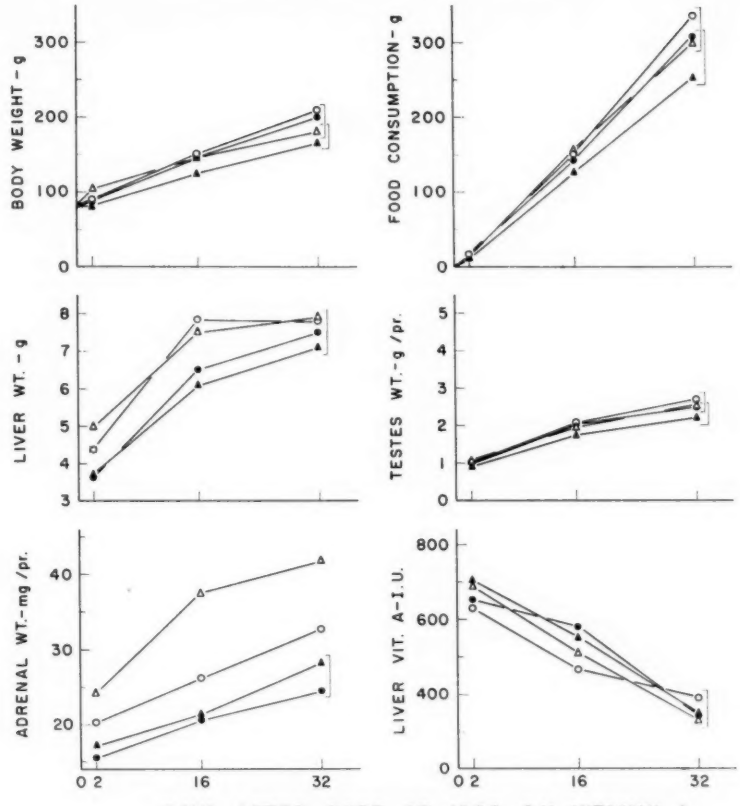

FIG. 1. Mean body weights, food consumption, liver, testes, and adrenal weights, and liver vitamin A of Wistar rats fed corn oil●, Wistar rats fed rapeseed oil▲, Sprague–Dawley rats fed corn oil○, and Sprague–Dawley rats fed rapeseed oil△, following a single dose of vitamin A.

as judged by the plateau of body weight, which was lower than that obtained with animals fed no fat or stearic acid. This observation, which again associated erucic acid with lower weight gain, might have been unrelated to vitamin A deficiency, since it is not clear that the onset of this condition occurred sooner with erucic acid in the diet. In fact, the decline in body weight appeared earlier in animals fed stearic acid. If rats receiving erucic acid have an increased requirement for vitamin A, such is not apparent when that fatty acid is a component of rapeseed oil, and the storage and depletion of vitamin A are used as the criteria for assessing the state of adequacy of the vitamin.

These studies with Wistar and Sprague–Dawley rats demonstrated a strain difference in the sensitivity of the weight gain response to two vegetable oils, in the apparent coefficients of digestibility, in the weights of testes and adrenals, and in total adrenal cholesterol, and no difference in the concentra-

tions of liver lipids and serum cholesterol, nor in the storage or depletion of liver vitamin A.

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AN ANOMALOUS TRICARBOXYLIC ACID CYCLE IN ACETOBACTER MELANOGENUM[1]

D. H. BONE[2] AND R. M. HOCHSTER

Abstract

Extracts of gluconate-grown *Acetobacter melanogenum* contain condensing enzyme and DPN-isocitric dehydrogenase of low specific activity. No evidence could be found for the presence of phosphotransacetylase, aconitase, or TPN-isocitric dehydrogenase. Since the organism or its extracts cannot synthesize the necessary four carbon compounds from pyruvate and from acetate, it is concluded that the tricarboxylic acid cycle does not function in extracts of this organism in the usually accepted manner.

The pyruvic oxidase system was found to be highly active, acetaldehyde being the chief intermediate and acetate the end product. The mechanism for the slow incorporation of acetate into other cell constituents is, at present, unknown.

Introduction

Previous work (1, 2) has demonstrated that cell-free preparations of *Acetobacter melanogenum* oxidize glucose to 2,5-diketogluconate via gluconate and 2-ketogluconate. These cell-free preparations were also found to oxidize 2,5-diketogluconate to α-ketoglutarate in the presence of the dye phenazine methosulphate (3). Katznelson (4) reported that glucose could also be phosphorylated and that the resultant hexose phosphate was oxidized predominantly to 6-phosphogluconate, followed by the splitting reaction of Entner–Doudoroff (5) to form pyruvate and glyceraldehyde-3-phosphate. The above two pathways of glucose oxidation by cell-free extracts produce two major intermediates which are of significance in the operation of the tricarboxylic acid cycle: pyruvate from hexose phosphate and α-ketoglutarate from 2,5-diketogluconate.

The present paper describes the results of studies on the metabolism of these substances and of other intermediates of the tricarboxylic acid cycle in this organism.

Materials and Methods

Materials

The following substances used in this investigation were commercial preparations: ATP,* ADP, AMP, IDP, ITP, TPN, DPN, and CoA (Pabst Brewing Co.); α-ketoglutaric acid, TRIS, lipoic acid, DPNH, TPNH, and

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[2]National Research Council of Canada Fellow in collaboration with the Canada Department of Agriculture, 1957–1959. Present address: Department of Microbiology, Yale University, School of Medicine, New Haven, Connecticut.

*The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; CoA, coenzyme A; TRIS, tris(hydroxymethyl)aminomethane; DPNH, reduced form of diphosphopyridine nucleotide; TPNH, reduced form of triphosphopyridine nucleotide; PEP, phosphoenolpyruvic acid; ThPP, thiamine pyrophosphate; *p*-CMB, *p*-chloromercuribenzoate; GSH, reduced glutathione; TCA, trichloroacetic acid; c.p.m., counts per minute, and m.p., melting point.

PEP (Sigma Chemical Co.); potassium gluconate (Pfizer); glyoxylic acid, isocitric acid, *cis*-aconitic acid (California Corporation for Biochemical Research); ThPP, *p*-CMB, oxaloacetic acid, fumaric acid, *l*-malic acid, sodium pyruvate (Nutritional Biochemicals Corporation); GSH (Schwarz Laboratories); succinic acid, citric acid, lactic acid, propionic acid, acetaldehyde (Fisher Scientific Co.); all C^{14}-labelled compounds (Radiochemical Centre, Amersham, England).

Oxaloacetate was assayed by decarboxylation with aluminum sulphate (6) and found to be 95% pure.

Methods

Cells of *A. melanogenum* (MA62) were grown in shake culture for 48 hours in a medium containing 2% potassium gluconate and 1% yeast extract. The cells were harvested, washed twice with distilled water, and suspended in three volumes of distilled water. They were then disrupted by sonic oscillation in a Raytheon 200 watt, 10 kc oscillator for 10 minutes at a power output of 1.0 amp. The resulting suspension was centrifuged for 10 minutes at 10,000×g at 4° C. Extracts were also prepared by grinding cells with twice their weight of alumina powder (Alcoa A-301) with the gradual addition of cold distilled water. The pestle and mortar were kept at 4° C. The suspension of cells and alumina was centrifuged at 10,000×g for 10 minutes at 4° C. Supernatant fluids were used for the assay of enzymic activities.

Extracts treated with Dowex-1(Cl^-) to remove CoA (7) were assayed for aceto-CoA-kinase (8), acetokinase (9), and phosphotransacetylase (10). Condensing enzyme activity was measured by incubating an extract with potassium phosphate, pH 7.4, 100 μmoles; GSH 10 μmoles; CoA, 0.15 μmole; $MgCl_2$, 10 μmoles; ATP, 10 μmoles; and sodium acetate-2-C^{14}, 5 μmoles (336,000 c.p.m.). The mixture was incubated at 30° C for 40 minutes and five 2-μmole aliquots of oxaloacetate were added at 8-minute intervals. The reaction was stopped by adding 3 ml of 95% ethanol, followed by centrifugation and washing of the precipitate with 1 ml of 70% ethanol. The combined supernatant solutions were evaporated to dryness at 40° C under a stream of air. To analyze for radioactive products, the residue was chromatographed on Whatman No. 4 paper using the butanol – propionic acid – H_2O solvent of Calvin and Benson (11). Radioactive compounds were located by scanning the paper with a mica end-window β-counter tube.

Enzyme reactions involving the reductions of DPN and of TPN, and the oxidations of DPNH and of TPNH, were carried out in anaerobic Beckman cuvettes by measuring the change in optical density at 340 mμ in a Beckman spectrophotometer (Model DU). Anaerobiosis was obtained by flushing the cuvettes with N_2 for 5 minutes followed by evacuation with a water pump for 15 minutes at 29 inches of mercury.

DPN-specific isocitric dehydrogenase was assayed according to the method of Kornberg and Pricer (12) with AMP omitted. For the determination of α-ketoglutaric dehydrogenase, the anaerobic cuvettes contained potassium phosphate buffer, pH 7.4, 100 μmoles; potassium α-ketoglutarate, 20 μmoles;

$MgCl_2$, 10 μmoles; GSH, 10 μmoles; lipoic acid, 0.3 μmole; ThPP, 0.3 μmole; DPN, 1.0 μmole; CoA, 0.2 μmole; 0.05 ml of extract; and water to 3.0 ml. Succinic dehydrogenase was assayed by measuring the reduction of methylene blue (13) at 660 mμ in anaerobic Beckman cuvettes. Fumarase was assayed by observing the change in absorption at 300 mμ in the presence of fumarate and extract (14). The oxidation of DPNH by oxaloacetate was used to assay malic dehydrogenase. Cuvettes contained potassium phosphate buffer, pH 7.4, 100 μmoles; oxaloacetate, 1 μmole; DPNH, 0.15 μmole; 0.05 ml of extract; and water to 3.0 ml.

The conventional Warburg technique was used for manometric determinations. Vessels contained 20 μmoles of substrate in 2.4 ml of 0.05 *M* potassium phosphate buffer, pH 7.4. In the case of acetaldehyde oxidase, 50 μmoles of acetaldehyde was placed in the center compartment. Protein concentration was estimated by the spectrophotometric method of Warburg and Christian (15). Experiments were carried out at 30° C unless specified otherwise.

Organic acids were separated from cell suspensions by first precipitating protein with 0.2 *N* perchloric acid and centrifuging, followed by neutralization of the supernatant with 2 *N* potassium hydroxide. The resultant potassium perchlorate was centrifuged down and an aliquot of the supernatant was analyzed for organic acids using a Dowex-1 formate column (16). Acetic acid was estimated in this protein-free extract with acetokinase (9). To estimate the C^{14} content of acetate, the protein-free extract was treated with 0.2 volume of 0.2% 2,4-dinitrophenyl hydrazine in 2 *N* HCl, followed by vacuum distillation of the acetic acid at 35° C. The latter was trapped in 0.2 *N* potassium hydroxide, and the specific activity of potassium acetate was measured. Keto acids were extracted as 2,4-dinitrophenyl hydrazones and freed of organic acids (17), followed by chromatography on Whatman No. 3 mm paper using *n*-butanol saturated with 6% ammonia (18) as developing solvent. Hydrazones were extracted with 0.05 *M* potassium phosphate buffer (pH 7.4), and estimated at 380 mμ for α-ketoglutarate and at 370 mμ for pyruvate. Hydrazones of α-ketoglutarate and of pyruvate were identified by reduction with H_2 (platinum oxide in glacial acetic acid) to form glutamate and alanine respectively.

Glutamic acid isolated from Dowex-1 formate columns was freed of radioactive impurities by paper chromatography with *n*-butanol – acetic acid – H_2O (4:1:2.5) as the solvent system. Radioactive glutamic acid was degraded as described by Handler and Anfinsen (19). Cells metabolizing acetate-2-C^{14} were fractionated with TCA, ether, and alcohol according to Roberts *et al.* (20). All radioactive samples were assayed as described by Moses (21) using a windowless gas flow (methane) proportional chamber connected to a scaler. Corrections were made for self-absorption.

Results

Oxidation of Tricarboxylic Acid Cycle Intermediates by Intact Cells

Washed suspensions of intact *A. melanogenum* cells oxidized only pyruvate; all other intermediates of the tricarboxylic acid cycle and glutamate were

not oxidized. The Q_{O_2} value (μl of O_2/mg dry wt./hour) for pyruvate oxidation was 60. Addition of tricarboxylic acid cycle intermediates to the growth medium did not facilitate their oxidation by the resulting resting cell suspensions. The oxidation of 1 mole of pyruvate resulted in the uptake of 1 atom of oxygen, the production of 1 mole of carbon dioxide, and of 1 mole of acetate.

Tricarboxylic Acid Cycle and Associated Enzyme Systems in Cell-free Extracts

Table I lists the specific activities of the enzymes of the tricarboxylic acid cycle which were found in cell-free extracts. Acetate was found to be activated

TABLE I

Specific activities of tricarboxylic acid cycle and associated enzyme systems in sonic, cell-free extracts of *A. melanogenum*

Enzyme	Specific activity, μmoles of substrate/hr/mg protein
Aceto-CoA-kinase	0.1
Condensing enzyme	0.02
Isocitric dehydrogenase (DPN)	0.1
α-Ketoglutaric dehydrogenase	0.8
Succinic dehydrogenase	0.1
Fumarase	0.9
Malic dehydrogenase	0.3
Oxaloacetic carboxylase	1.1
Pyruvic oxidase	4.4
Pyruvic oxidase (TPN)	1.7
α-Carboxylase	4.3
Acetaldehyde oxidase	6.1

by aceto-CoA-kinase. In order to demonstrate the dependency of the reactions on CoA, extracts were treated with Dowex-1(Cl^-) resin according to Chantrenne and Lipmann (7). Table II illustrates the clear-cut CoA and

TABLE II

Activation of acetate by an extract treated with Dowex-1(Cl^-)

Conditions	Acetohydroxamic acid formed in 1 hour, μmoles
Complete system	1.10
Complete system, KF omitted	1.56
Complete system, acetate omitted	.28
Complete system, CoA omitted	.22
Complete systems, ATP omitted	.22
Complete system, Mg^{++} omitted	1.16

NOTE: Components (in 1.0 ml): tris buffer, pH 7.4, 50 μmoles; potassium acetate, 20 μmoles; CoA, 0.2 μmole; ATP, 10 μmoles; $MgCl_2$, 10 μmoles; KF, 50 μmoles; GSH, 10 μmoles; hydroxylamine, 200 μmoles; extract, 16 mg protein, previously treated with Dowex-1(Cl^-).

ATP requirements for the formation of acetohydroxamic acid. It may be inferred that acetokinase is probably not functioning in these extracts. Furthermore, no evidence could be found for phosphotransacetylase.

As shown in Table III, the formation of radioactive citrate by the condensing enzyme present in the extracts was found to be dependent on Mg^{++}, ATP,

TABLE III

Activity of condensing enzyme in extracts prepared by alumina grinding

Conditions	Citrate formed, c.p.m.
Complete system	34,744
Complete system, with boiled extract	144
Complete system, oxaloacetate omitted	160
Complete system, Mg^{++} omitted	2,911
Complete system, ATP omitted	160
Complete system, CoA + GSH omitted	840

NOTE: Components of reaction mixture: see under "Methods"; extract, 27 mg protein.

CoA, GSH, acetate, and oxaloacetate. Alumina-ground extracts, sonic extracts, and extracts prepared in the presence of 0.05 *M* tris buffer (pH 7.4) and 0.01 *M* GSH were found to have the same activity of condensing enzyme (0.02 μmole/hour/mg protein). The radioactive citrate formed was identified by cochromatography with authentic citrate in phenol – formic acid (22), *n*-butanol – propionic acid – H_2O (11), and *t*-amyl alcohol – chloroform – formic acid – H_2O (23).

A DPN-specific isocitric dehydrogenase was found in the extracts which was not activated by AMP, a previously recognized characteristic of the yeast enzyme (12). Extracts oxidized isocitrate at a rate equivalent to that of the DPN-specific isocitric dehydrogenase and took up 1 μatom of oxygen per μmole of isocitrate. Chromatograms of the 2,4-dinitrophenyl hydrazones isolated from the reaction mixture showed only the presence of the hydrazone of α-ketoglutaric acid.

Extracts did not reduce DPN in the presence of α-ketoglutarate directly but when the extract was supplemented with CoA, ThPP, ATP, Mg^{++}, and lipoic acid, DPNH formation was observed. Sodium arsenite (10^{-3} *M*) was found to inhibit α-ketoglutaric dehydrogenase completely. Oxaloacetic decarboxylase was also present. Appropriate control experiments were carried out for the non-enzymatic breakdown of oxaloacetate which was found not to be affected by ATP.

The pyruvic oxidase system was found to be one of the most active enzyme systems related to the tricarboxylic acid cycle in *A. melanogenum* (Table I). Pyruvate appeared to be metabolized by extracts in the same manner as by whole cells, the main products of oxidation being equimolar quantities of acetate and of carbon dioxide (see "oxidation of tricarboxylic acid cycle intermediates by intact cells"). The rate of pyruvate oxidation by an extract was not affected by the addition of ATP, CoA, ThPP, DPN, and TPN. In order to demonstrate cofactor requirements, extracts were treated with ammoniacal ammonium sulphate according to Green *et al.* (24) and the fraction between 30%–70% saturation was collected by centrifugation. The precipitate so formed was washed with neutral, saturated ammonium sulphate solution and was used in the experiment described in Table IV. The maximal initial rate of oxidation of pyruvate was dependent on the presence of Mg^{++}, ThPP, DPN

TABLE IV

Pyruvic oxidase in the ammoniacal ammonium sulphate fraction

Conditions	O_2 uptake in 30 minutes, μl
Complete system	126
Complete system, Mg^{++} omitted	98
Complete system, ThPP omitted	75
Complete system, DPN omitted	40
Complete system, CoA omitted	113
Complete system, GSH omitted	77
Complete system, ATP omitted	111
Complete system, TPN replacing DPN	86
System without any added cofactors	7

NOTE: Components (in 2.4 ml): potassium phosphate, pH 7.4, 120 μmoles; sodium pyruvate, 20 μmoles; $MgCl_2$, 10 μmoles; ThPP, 0.2 μmole; DPN, 1 μmole; TPN, 1 μmole; CoA, 0.15 μmole; GSH, 10μmoles; ATP, 5 μmoles; ammoniacal ammonium sulphate fraction (30%–70%), 0.5 ml.

or TPN, and GSH. CoA and ATP had only a very slight effect on pyruvate oxidation. Extracts which had been treated with Dowex-1(Cl^-) for assay of aceto-CoA-kinase were found to require ThPP and DPN or TPN for pyruvate oxidation, but not CoA.

The effects of various inhibitors on pyruvate oxidation were also studied and the results are given in Table V. Dimedone, a specific reagent for aldehydes, inhibited only oxygen uptake; this suggests that an aldehyde is probably an intermediate in the oxidation of pyruvate.

TABLE V

Effects of inhibitors on pyruvate metabolism

Inhibitors	Inhibition of O_2 uptake, %	Inhibition of CO_2 output, %
Dimedone	45	0
Sulphathiazole	100	100
Arsenite + GSH	7	0
p-CMB	100	100

NOTE: Components (in 2.4 ml): potassium phosphate buffer, pH 7.4, 120 μmoles; sodium pyruvate, 20 μmoles. Additions: dimedone, 30 μmoles; sulphathiazole, 12 μmoles; sodium arsenite, 2.4 μmoles; GSH, 10 μmoles; *p*-CMB, 0.24 μmole; extract, 18 mg protein. Duration of experiment, 20 minutes.

Sulphathiazole and *p*-CMB, inhibitors of yeast α-carboxylase (24, 25), effectively inhibited the metabolism of pyruvate in extracts of *A. melanogenum*. Arsenite inhibited oxygen uptake only slightly in contrast to its ability to inhibit α-ketoglutaric dehydrogenase completely. To obtain inhibition of pyruvate oxidation by arsenite, it was essential to first add GSH to the extract, followed by arsenite (26). A similar effect was noted with the acetaldehyde oxidase system to be described below.

As dimedone was found to be an inhibitor of pyruvic oxidase (27) and thus acetaldehyde could be considered as a possible intermediate in the oxidation of pyruvate, air was blown over the surface of the extract during pyruvate oxidation (at 30° C), and the vapors were passed through two separate outlets

into: (1) a saturated solution of dimedone in water and (2) through 0.2% 2,4-dinitrophenyl hydrazine in 2 *N* HCl. Derivatives were thus obtained and recrystallized from dilute alcohol and alcohol respectively. The dimedone derivative (m.p. 142° C) was identical with that of an authentic acetaldehyde dimedone compound (m.p. 142° C) as a mixed m.p. of 142° C was obtained. Similarly, the 2,4-dinitrophenyl hydrazones were found to be acetaldehyde derivatives (m.p. of unknown hydrazone, 166° C; acetaldehyde hydrazone, 164° C; mixed m.p. 163° C).

Extracts were found to form carbon dioxide from pyruvate under anaerobic conditions, and dimedone was found to increase the formation of carbon dioxide by 90%. This is interpreted as resulting from acetaldehyde removal (as the dimedone compound) thus freeing the system from acetaldehyde inhibition (24). The participation of α-carboxylase as a component of the pyruvic oxidase system is thus demonstrated indirectly. An ammoniacal ammonium sulphate fraction (30%–70% saturation) was assayed for α-carboxylase activity. Table VI shows that carbon dioxide production required the presence of Mn^{++} ions and ThPP.

TABLE VI

Effect of thiamine pyrophosphate and of manganese ions on the decarboxylation of pyruvate

Conditions	CO_2 production in 30 minutes, μl
Pyruvate	0
Pyruvate + Mn^{++}	24
Pyruvate + ThPP	63
Pyruvate + Mn^{++} + ThPP	72

NOTE: Components (in 2.4 ml): sodium pyruvate, 20 μmoles; $MnSO_4$, 1 μmole; ThPP, 0.2 μmole; Tris buffer, pH 7.4, 120 μmoles; dimedone, 30 μmoles; ammoniacal ammonium sulphate fraction (30%–70%), 0.5 ml. Atmosphere: 95% nitrogen+5% carbon dioxide.

Acetaldehyde was actively oxidized by cell-free extracts with a specific activity of 6.1 μmoles substrate/hour/mg protein (Table I). The main product of the oxidation was acetate as determined by acetokinase assay (9). The addition of the cofactors DPN, TPN, CoA, GSH, and Mg^{++} did not affect the activity of acetaldehyde oxidase in whole extracts. Acetaldehyde oxidase activity in extracts treated with Dowex-1(Cl^-) was, however, found to be stimulated 12% by DPN and 25% by TPN. CoA, GSH, and Mg^{++} had no effect under these conditions. The inhibition (11%) of acetaldehyde oxidase by 10^{-3} *M* arsenite required the prior incubation of the enzyme with GSH. The reduction of pyridine nucleotides by acetaldehyde dehydrogenase was difficult to measure, especially in the anaerobic Beckman cuvettes used, owing to the volatile nature of acetaldehyde.

Pyruvate reduced TPN only under anaerobic conditions, while pyruvic oxidase (TPN) could account for 39% of the activity of the pyruvic oxidase system as measured manometrically (see Table I). No net DPN reduction was observed when pyruvate was the substrate. This is explained by the presence

of a DPN-specific lactic dehydrogenase and of alcohol dehydrogenase which, together, probably maintain DPN in its oxidized form.

No evidence could be obtained for the presence of aconitase (28), TPN-specific isocitric dehydrogenase (29), malic enzyme (30), Utter–Kurahashi enzyme (31), phosphoenolpyruvic carboxylase (32), malate synthetase (33), and of isocitritase (34) in extracts prepared under a variety of conditions.

All attempts made to obtain a net synthesis of four carbon C^{14}-compounds gave negative results. In these experiments extracts were incubated with the required combinations of $HC^{14}O_3$, pyruvate-2-C^{14}, acetate-2-C^{14}, lactate, propionate, PEP, glyoxylate, oxaloacetate, Mg^{++}, GSH, CoA, AMP, ADP, ATP, ITP, IDP, DPN, TPN, DPNH, and TPNH. However, there was an exchange reaction between $HC^{14}O_3$ and oxaloacetate which had a specific activity of 0.03 mμmole of carbon dioxide fixed/hour/mg protein. A similar exchange reaction has been described for the oxaloacetate decarboxylase of *Micrococcus lysodeikticus* (35).

Metabolism of C^{14}-labelled Acetate, Bicarbonate and Pyruvate by Whole Cells

In order to establish whether pyruvate was oxidized beyond the acetate stage, cells were incubated with pyruvate-2-C^{14} under aerobic conditions. The perchloric acid soluble fraction was analyzed for organic acids, and the results are given in Table VII. Pyruvate-2-C^{14} was oxidized mainly to acetate (85% of initial pyruvate) with smaller amounts of C^{14} incorporated into α-ketoglutarate and glutamate. Comparison of the specific activities of acids isolated indicate that there was incomplete isotopic equilibration under the experimental conditions used. When glutamic acid isolated from this experiment was degraded (19), 7.9% of the radioactivity was found in carbon-1, 90.2% occurred in carbon-5, and no C^{14} was detected in carbons 2, 3, and 4. When cells were incubated with acetate-1-C^{14} a similar C^{14}-labelling pattern in glutamate resulted.

TABLE VII

Incorporation of pyruvate-2-C^{14} by resting cell suspensions

Compound	Radioactivity, c.p.m.	Quantity, μmoles	Specific activity, c.p.m./μmole
Citrate	14,435	—	—
α-Ketoglutarate	303,300	1.5	202,200
Glutamate	780,400	3.9	200,100
Succinate	1,584	—	—
Malate	1,188	—	—
Pyruvate	821,800	6.7	122,650
Acetate	17,040,000	43.2	394,440
Carbon dioxide	202,880	56.4	3,600

NOTE: Components (in 35 ml): potassium phosphate buffer, pH 7.4, 400 μmoles; sodium pyruvate-2-C^{14}, 50 moles (400,000 c.p.m./μmole); cells, 7.3 g wet weight. Duration of experiment, 15 minutes.

Cells growing in the exponential phase were found to incorporate a small amount of acetate-2-C^{14} into some cell materials. The distribution of C^{14} obtained by the use of the method of Roberts *et al.* (20) is shown in Table VIII. It is readily seen that the bulk of acetate-2-C^{14} contained within the cells was

TABLE VIII

Incorporation of acetate-2-C^{14} by exponentially growing cells

Components examined	Radioactivity, c.p.m.	% of initial acetate
Carbon dioxide	5,000	0.025
Cells	1,823,980	9.1
Neutralized cold-TCA soluble	1,256,600	6.28
Fractions		
Cold TCA soluble	38,564*	—
Alcohol–ether soluble	171,174	—
Alcohol soluble – ether insoluble	81,810	—
Hot-TCA soluble	26,600	—
TCA insoluble	172,284	—
Sum of fractions	490,432	2.45

NOTE: 1 liter of medium containing 20 g potassium gluconate; 10 g yeast extract; sodium acetate-2-C^{14}, 10 μmoles (2×10^8 c.p.m./μmole). Duration of experiment, 5 hours, temperature, 27° C.
*When sample is not neutralized, free C^{14} acetic acid readily volatilizes under the drying conditions employed in the preparation of planchettes, prior to counting.

in the form of free acetate-2-C^{14} (trapped in the neutralized cold-TCA soluble fraction). Acetate-2-C^{14} was utilized for lipid synthesis (alcohol–ether soluble) and for the synthesis of protein and cell wall constituents (TCA insoluble). Hydrolysis (acetic acid and concentrated HCl (1:1) for 18 hours at 100° C) of the TCA insoluble fraction and separation of the amino acids on a Dowex-50 column revealed that 60% of the C^{14} was located in the glutamate residues, whereas negligible C^{14} was found in the other protein amino acids.

The incorporation of $HC^{14}O_3$ into exponentially growing cells was found to be negligible. Conditions of the experiment were the same in this case as those given in Table VIII, except that sodium acetate-2-C^{14} was replaced by sodium bicarbonate (31.5 μmoles containing 5×10^7 c.p.m.). Cells were found to have incorporated 1,500 c.p.m. of $HC^{14}O_3$.

Discussion

The work reported here has shown that gluconate-grown *A. melanogenum* contains condensing enzyme and DPN-isocitric dehydrogenase which exhibit very low activities, especially when these are compared with the activities of other enzymes, notably those catalyzing the conversion of α-ketoglutarate to other tricarboxylic acid cycle intermediates. Furthermore, aconitase activity could not be demonstrated at all. These facts suggest that the tricarboxylic acid cycle in extracts of this organism presents a picture which differs considerably from that usually encountered.

Absence of the condensing enzyme has been reported for *Acetobacter suboxydans* (36) and *Clostridium butylicum* (37), but the assay procedure used was limited by the sensitivity of the chemical determination of citrate which is much lower than the highly sensitive radioactive method used here. The presence of a DPN-linked isocitric dehydrogenase and the absence of the corresponding TPN-linked enzyme in our extracts is also unusual. This is especially noteworthy since the TPN-enzyme is usually the more active and stable and the DPN-catalyzed system is quite a labile enzyme in most biological tissues.

The pyruvate oxidase system consists of at least two enzymes, α-carboxylase and acetaldehyde oxidase. The rate limiting step is probably α-carboxylase as judged from the relative activities of the enzymes (Table I), *K*m values (pyruvic oxidase, 10^{-2} *M*; α-carboxylase, 10^{-2} *M*; acetaldehyde oxidase, 4×10^{-3} *M*), and the absence of acetaldehyde in extracts oxidizing pyruvate at 14° C (38).

The two accepted functions of the tricarboxylic acid cycle are the synthesis of carbon skeletons for protein and nucleic acid precursors from acetyl-CoA and oxaloacetate and also the oxidation of intermediates of the cycle to yield carbon dioxide and water with the concomitant formation of ATP. In the case of *A. melanogenum*, the net synthesis of intracellular cell materials from pyruvate is not very significant owing to the absence of enzymes capable of synthesizing oxaloacetate from pyruvate or a closely related compound and due to the slow conversion of acetyl-CoA to α-ketoglutarate via the condensing enzyme. Experiments with labelled acetate, bicarbonate, and pyruvate show that acetate can be slowly incorporated into cell constituents, the mechanism being, at present, unknown. It is possible that this involves endogenous substrate material since it has been stated that extracts of this organism and intact cells are incapable of synthesizing the necessary four-carbon precursors required in the biosynthesis of cell materials. This is further illustrated by the similar labelling pattern of glutamate derived either from acetate-1-C^{14} or from pyruvate-2-C^{14}.

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ELECTROLYTE METABOLISM IN THE RAT EXPOSED TO A LOW ENVIRONMENTAL TEMPERATURE. II[1]

D. G. BAKER

Abstract

Rats acclimatized to cold (2° C) have lower subcutaneous tissue and superficial muscle temperatures than comparable animals living at 25° C. The total water content of the skin is significantly increased after acclimatization to cold. This increase is due to an increase in the chloride space. The total water content of muscle was decreased in the rats acclimatized to cold although the chloride space (extracellular water) was increased. The intracellular sodium of muscle was decreased by exposure to cold.

Baker and Sellers (2) reported that when rats were exposed to a low environmental temperature there was an increase in the concentration of sodium in the plasma. A sodium retention, without a concomitant chloride retention, and an increase in total body water were also found. The authors suggested that this was due, in part, to the increased metabolic activity in the tissues of animals exposed to cold. They also suggested that the sodium retention could have been due to an increased sodium space in the peripheral tissues, which presumably would be at a lower temperature in those animals exposed to cold. This report described work which was undertaken in order to test the latter hypothesis.

Materials and Methods

Male rats of the Wistar strain (Carworth Farms) and weighing 180 to 225 g were used. All animals were kept in individual cages and offered Fox Breeder cubes (Toronto Elevators) and tap water ad libitum. Rats acclimatized to cold had been maintained in a cold room (1° ± 2° C) for a period of approximately fifty days.

The plan of the experiment was first to determine the temperatures of the subcutaneous tissues of the back, as an example of subcutaneous tissue, and of the gastrocnemius muscle, as an example of superficial muscle. Total water $(H_2O)_T$, sodium $(Na)_T$, and chloride $(Cl)_T$ determinations (10) were then made on tissues from these regions to determine if a correlation between temperature and electrolyte composition could be demonstrated.

The tissue temperature measurements were made as follows: Copper–constantan thermocouples were prepared from No. 30 55-1 wire. These were fine enough to slide down a No. 18 syringe needle. The thermocouple temperatures were registered on a Leeds and Northrup recording potentiometer (Speedomax mod. 60000, type G). The rat was held by an assistant while the point of the syringe needle was placed in the tissue to be measured. The thermocouple was then inserted through the needle and the shaft of the needle withdrawn leaving the thermocouple implanted in the tissue. The temperature

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was recorded at about half-minute intervals until constant readings were obtained (1 to 4 minutes).

Temperature measurements were made on each of the rats acclimatized to cold while they were still in the cold room. Each animal was then removed from the cold room and the temperatures again measured. With this procedure, the cold-acclimatized rats were at room temperature (25° C) for 3 to 4 minutes. Similarly, the temperatures of the tissues of control rats were measured at room temperature and again after being in the cold for 3 to 4 minutes. This crossover type of experimental design was used to facilitate the statistical analysis of the data. Twenty-two rats acclimatized to cold and a similar number of rats from the same original stock, but living at an environmental temperature of 25° C, were examined.

The procedure for the chemical determinations was as follows: The fur was removed from the back and hind legs of the rat by electric clippers. The animal was then killed by a blow on the neck, and five samples of muscle from the rear limbs were removed and trimmed free from excess fat and connective tissue. Five samples of shaved skin were also obtained. All the tissue samples were dried to constant weight at 92 to 95° C and the average of the weight losses used to calculate the total water content, $(H_2O)_T$, of the respective tissues. Two dry samples of each tissue were subjected to acid digestion and the sodium content of the digested material was determined using a Beckman DU Spectrophotometer with a "flame" attachment. Care was taken to use standards with similar anion and cation concentrations. The remaining three samples permitted triplicate determinations of chloride concentration to be made using a modified Van Slyke procedure (9).

Calculations

Where values for the concentrations of plasma electrolytes were required in order to make "space" calculations, the average values previously reported (2) were used. It must be pointed out that, although these same animals (2) were used for the present study, the blood samples were obtained a week or more prior to the time when the tissues were taken for analysis. It was felt that such a procedure was justified since (*a*) the averages were based on a large number of animals and (*b*) a relatively small variation was found between animals within each environmental group.

$$\text{Chloride space}^* \ (H_2O)_E^{Cl} = \frac{(Cl)_T}{(Cl)_s} \times (H_2O)_s \times r_{Cl}$$

where $(Cl)_T$ = meq of chloride per kg tissue,
$(Cl)_s$ = meq of chloride per kg serum,
$(H_2O)_s$ = grams of water per kg serum,
r_{Cl} = Gibbs–Donnan ratio for chloride = 0.98,
$(Na)_c$ = concentration of intracellular sodium in meq per kg tissue.

*The symbols and method of calculation follow those used by J. F. Manery. Physiol. Rev. **34**, 334 (1954).

Results

The temperature measurement data are presented in summary in Table I, with the analyses of variance in Table II. From these it is apparent that the animals acclimatized to cold (2° C) had lower subcutaneous tissue and superficial muscle temperatures than comparable animals living at 25° C. Periods of time less than five minutes at 25° C were sufficient to produce a small but significant change in the subcutaneous temperature of the cold-acclimatized rats, but not in the temperature of the muscles.

TABLE I

Average temperature of the subcutaneous and muscle tissues of rats acclimatized to 2° C or 25° C*

	Acclimatized to cold, measured at:		Normal measured at:	
	2° C	25° C	2° C	25° C
Subcutaneous	34.9	35.8	36.2	36.3
Muscle	34.7	35.1	38.0	37.2

*Measurements were made while the animals were at each environmental temperature.

TABLE II

Analysis of variance of the temperature measurements for muscle and subcutaneous tissues

		Muscle tissue		Subcutaneous tissue	
Source of variation	D.f.	S.s.	M.s.	S.s.	M.s.
Total	87	362.10		274.89	
Between animals	42	126.92	3.02	172.82	4.11
Cold (C) vs. normal (N)	1	162.00	162.00*	16.85	16.85*
Inside (I) vs. outside (O)	1	0.62	0.62	76.11	76.11*
Interaction (C) (N) with (I) (O)	1	6.99	6.99*	27.34	27.34*
Error	42	65.57	1.56	11.11	0.26

*Statistical significance (F test) showing $P < .05$.
Inside (I) or outside (O) indicates that the temperature of the tissue was measured inside (I) or outside (O) of the cold room.

The data obtained from the chemical analysis of the skin and muscle are summarized in Table III. The plasma composition used for the space calculations is shown in Table V.

The total water content of skin $(H_2O)_T$ was significantly increased after acclimatization to cold. This increase was due to an increase in the chloride space $(H_2O)_E^{Cl}$, presumably extracellular. The total water content of muscle was decreased after acclimatization to cold.

If we assume that the chloride space is extracellular and has the same sodium ion concentration per gram of water as the plasma then, by using the values of $(Na)_T$ from Table III, one is able to calculate the concentration of intracellular sodium (Table IV). With regard to muscle, this was decreased in the animals exposed to cold. In skin, the sodium space $(H_2O)_E^{Na}$ was only slightly increased after acclimatization to cold while the chloride space

TABLE III

Average sodium, chloride, and water composition of muscle and skin

Test		Environmental temperature 2° C	25° C
$(Na)_T$, meq/kg	Skin	66.42 ± 0.98	64.23 ± 1.24
	Muscle	28.15 ± 1.09	26.93 ± 0.94
$(Cl)_T$, meq/kg	Skin	54.89 ± 4.72	52.38 ± 4.21
	Muscle	15.13 ± 2.82	12.56 ± 0.60
$(H_2O)_E^{Cl}$, g/kg	Skin	563.2 ± 4.8	549.0 ± 3.5*
	Muscle	156.5 ± 2.9	130.3 ± 6.2*
$(H_2O)_T$, g/kg	Skin	620.9 ± 3.1	602.1 ± 2.5*
	Muscle	750.5 ± 2.6	759.9 ± 2.7*

*Statistically significant difference $P < .05$.
Results shown are based on samples from 11 to 18 rats for each value. Sodium and chloride concentrations are expressed as meq per kg of fresh weight and water is expressed in g per kg of fresh weight ± the standard error.

TABLE IV

Estimations of sodium space and of the intracellular concentration of sodium in muscle

Test		Environmental temperature 2° C	25° C
$(H_2O)_E^{Na}$, g/kg	Skin	432	427
	Muscle	183	179
$(Na)_e$ of muscle in meq per kgm of intracellular water		3.0	6.5

TABLE V

Plasma composition*

	2° C	25° C
Na, meq/liter	151.9	148.4
Cl, meq/liter	101.4	101.3
Specific gravity	1.026	1.026

*From Baker and Sellers (2). Average values based on 20 samples from rats in each environmental temperature.

$(H_2O)_E^{Cl}$ was increased to a greater extent. Thus a larger proportion of the chloride space was unavailable for sodium ions.

The chloride space in both the skin and muscle of the rats acclimatized to cold was increased. The fact that the sodium space of these same tissues remained practically unaltered suggests that some explanation other than that they contained more blood ultrafiltrate must be sought. An investigation of this interesting finding has been initiated.

The concentration of sodium and chloride in both the skin and muscle of animals acclimatized to cold was greater than that observed in normal animals. This increase, however, was not statistically significant.

Using the chloride space of muscle as a measure of extracellular water, it can be inferred that this is increased in the cold by 26 g per kg, whereas the

total water is decreased by 10 g per kg of fresh muscle. From this it can be calculated that the intracellular water was decreased by 35 g per kg. Similarly it can be shown that there is 4.6 g per kg less intracellular water in the skin of rats acclimatized to cold. If there is a relative cellular dehydration, it might be an adaptive mechanism as it would tend to make cells more resistant to cold injury from freezing. Such a mechanism is probably operative in insects (11) and other poikilotherms.

Microscopic examination of the skin and muscle from the rats acclimatized to cold and from their controls was made. Sections stained with hematoxylin and eosin for fat and connective tissue failed to show well-defined differences between these tissues from the two groups of animals. However, there was a suggestion of a decrease in thickness of the epidermis in the rats acclimatized to cold.

In order to define the tissue analyzed as skin a photomicrograph of the tissue is illustrated in Fig. 1.

FIG. 1. Photomicrograph of a representative piece of skin from a rat acclimatized to cold. Haematoxylin and eosin. ×40.

Discussion

The reduced subcutaneous temperature of the rat acclimatized to cold is suggestive of a reduced blood flow in the peripheral vessels. The fact that after only 4 minutes of exposure to 25° C the subcutaneous temperature of these animals was significantly elevated suggests that the temperature reduction was due, in part, to cold-induced vasoconstriction.

The temperature of the gastrocnemius muscle was lower in the rats acclimatized to cold. No change in the temperature of this tissue was observed after about four minutes at 25° C. Perhaps this was too brief a time to permit the vascular tone in vessels of the deeper tissues to respond to the increase in environmental temperature, but the possibility of a more permanent vascular response to cold must not be overlooked. In the Eskimo, who presumably is acclimatized to cold, Brown and his collaborators (4, 5) have investigated peripheral blood flow and tissue temperatures. They report that although the surface temperatures are higher on the bared hand and forearm of the Eskimo, the deep forearm temperatures are lower than in the non-adapted person at the same ambient temperature. An increase in skin temperature has been reported by Carlson *et al.* (6) whose subjects were permanent inhabitants of the Arctic. The lower muscle temperature observed in the rats acclimatized to cold is surprising in that their deep body temperatures (rectal) are, on the average, over a degree higher (3) than that found in rats living at 25° C.

Possibly the mechanism of acclimatization to cold involves an adaptation of metabolic processes so that they can function efficiently at a reduced temperature. Des Marais (7) observed an increase in the succinic dehydrogenase activity, measured at 37° C, of the skin in animals exposed to cold. From this he concluded that exposure to cold resulted in an increased metabolic activity of the skin. It would be interesting to know whether the increase would have been observed had the measurements been made at the in vivo temperatures of the skin.

The total water content of superficial muscle, in contrast to that of the skin, was reduced in the rats acclimatized to cold. However, as in the skin, the chloride space of the muscle (presumably extracellular) was increased, suggesting a relative cellular dehydration in this tissue in rats which had been acclimatized to cold. There is evidence (1, 8) which suggests that the water and electrolyte composition of tissue is maintained, in part, by a thermosensitive series of metabolic processes.

The sodium content of the tissues analyzed would not account for all of the sodium retention described in our previous report.

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THE FLUORESCENCE OF EIGHT NATURAL ESTROGENS IN SULPHURIC ACID[1]

WILLIAM S. BAULD,[2] MORRIS L. GIVNER,[3] LEWIS L. ENGEL,[4] AND JOSEPH W. GOLDZIEHER[5]

Abstract

The sulphuric acid fluorescence and absorption spectra of eight natural estrogens (estrone, estradiol-17β, estriol, 16-epiestriol, 16-ketoestradiol-17β, 16α-hydroxyestrone, 16-ketoestrone, and 2-methoxyestrone) have been studied with respect to the influence of exciting radiation wavelength, time of heating, acid concentration, and the addition of ethanol or toluene–ethanol. These data are evaluated with respect to optimum fluorescence intensity and specificity.

Introduction

A survey of fluorescence of the three classical estrogens in sulphuric and phosphoric acids has been published (4).

The influence of sulphuric acid concentration on the fluorescence of estrone, estradiol-17β, and estriol has been extensively investigated using fluorometers with interference filters (3, 7, 9). However, there is disagreement among the investigators on the optimum conditions for maximum fluorescence. This may be due in part to differences in the secondary filters of their fluorometers (7, 9). With the development of a precision spectrofluorometer of high resolving power (14), it was decided to use this instrument to establish the optimum conditions for the fluorescence of eight natural phenolic estrogens in sulphuric acid. The study was divided into three parts: first, to investigate more fully the fluorescence and absorption spectra of estrone, estradiol-17β, and estriol in aqueous sulphuric acid; second, to determine the fluorescence and absorption spectra of 16-epiestriol, 16α-hydroxyestrone, 16-ketoestradiol-17β, 16-ketoestrone, and 2-methoxyestrone in aqueous sulphuric acid; and finally, to establish the optimum conditions for the fluorometric estimation of these eight natural estrogens.

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[2]Department of Metabolism, The Montreal General Hospital, Montreal, Que. Dr. Bauld died July 20, 1958.
[3]The Department of Metabolism and the McGill University Clinic, The Montreal General Hospital, Montreal, Que.; Fellow of the Salmon Foundation For Medical Research Inc.; work supported in part by grants from National Research Council of Canada, Maurice Pollack Foundation, and David L. Harrington, Esq. Present address: Department of Endocrinology, The Southwest Foundation for Research and Education, San Antonio, Texas.
[4]Permanent Faculty Fellow of the American Cancer Society, The John Collins Warren Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. The portion of the work done in these laboratories was supported by grants from the National Cancer Institute, United States Public Health Service, a grant from the American Cancer Society, Inc., and a grant from the Jane Coffin Childs Memorial Fund for Medical Research.
[5]Department of Endocrinology, The Southwest Foundation for Research and Education, San Antonio, Texas.

Materials and Methods

Materials

Chromatographically pure, crystalline estrone, estradiol-17β, estriol, 16-epiestriol, 16α-hydroxyestrone, 16-ketoestradiol-17β, 16-ketoestrone, and 2-methoxyestrone were used in this study. Standard solutions of the eight estrogens were prepared in redistilled ethanol. Portions containing 10 μg of estrogen were pipetted into test tubes and the ethanol evaporated in a boiling-water bath. The 10-μg portion was chosen to permit accurate absorption and fluorescence measurements on the same sample.

Ethanol (96% by weight) was allowed to stand for a week with *m*-phenylenediamine and was distilled twice in an all-glass system.

Baker's sulphuric acid (reagent grade) was purified further by *in vacuo* distillation (13). Dilutions of sulphuric acid were prepared on a volume per volume basis (v/v). Thus for a 60% (v/v) solution, 60 ml of concentrated sulphuric acid (sp. gr., 1.84) was diluted to 100 ml with water. The acid concentrations were determined by specific gravity (hydrometer) and molarity (titrations with sodium hydroxide solutions) measurements.

The toluene (Eastman Kodak, sulphur-free reagent grade) was shaken with 6×0.1 volume of concentrated sulphuric acid for 30 minutes per volume. This was followed by successive washings with water, Na_2CO_3 (1 *N*), water, and distillation. The distilled toluene was allowed to stand overnight with concentrated sulphuric acid (0.1 volume) and finally washed and distilled as above.

Apparatus

Absorption and fluorescence spectra were determined on the same sample with a ratio recording spectrophotometer (Beckman DK-2) and a recording spectrofluorometer (instrument II) which has been described elsewhere (14).

A Farrand fluorometer (Model A) was used for some quantitative studies on the effect of toluene–ethanol and the time of heating on fluorescence intensity. The primary (lamp) filter was a combination of a Baird 436 mμ interference filter, a Corning No. 5113 glass filter, and a Polaroid film (HN38). Three photocell filter systems were used. One consisted of a combination of a Baird 492 mμ interference filter, a Corning 5030 glass filter, and a crossed Polaroid film. The second combination consisted of a Baird 529 mμ interference filter and the Polaroid film. The third combination consisted of a Baird 573 mμ interference filter and the Polaroid film. The transmission characteristics of the interference filters were determined with a Beckman model DU spectrophotometer. Estrone, estradiol-17β, 16-epiestriol, and estriol were studied with the first combination, 16α-hydroxyestrone with the second, and 2-methoxyestrone with the third.

General Procedure

Pure estrogens were heated in a boiling-water bath for 10 minutes with 3 ml of aqueous sulphuric acid with or without the addition of ethanol or toluene–ethanol (19/1, v/v). The test tubes were shaken after 1 and 2 minutes of heating. At the completion of the heating time they were placed in an ice bath for 5 minutes and at room temperature for at least 30 minutes. All readings

were carried out against a reagent blank. For relative fluorescence intensity measurements, the estrogen solution was compared to 11.2 μg estrone in 3 ml 80% (v/v) H_2SO_4 rendered fluorescent as described previously.

Results

Effect of Exciting Wavelength on Fluorescence Spectra

Estrone (1 μg in 80% (v/v) H_2SO_4) was irradiated with 365-, 405-, and 436-mμ mercury lines. The 436-mμ incident light was the most effective in producing a high fluorescence intensity. With these three mercury lines, the fluorescence peak was at 480 mμ. In a similar experiment, estradiol-17β (10 μg in 80% (v/v) H_2SO_4) was irradiated with light at these three wavelengths. A noteworthy effect was a pronounced increase in the 445-mμ fluorescence "shoulder" of estradiol-17β with incident light of 405 mμ when compared with excitation at 365 or 436 mμ. The fluorescence peak of estradiol-17β under these conditions was at 480 mμ. Irradiation of estriol (10 μg in 80% (v/v) H_2SO_4) with light of 480 mμ resulted in a broad fluorescence spectrum ranging from 500 to 600 mμ with a slight rise from 530 to 550 mμ. With 436-mμ incident light, this estriol solution showed a fluorescence peak at 480 mμ. 16α-Hydroxyestrone (10 μg in 88% (v/v) H_2SO_4) was irradiated with light at 365, 405, 436, and 492 mμ. The fluorescence peak was at 529 mμ, when the 365, 405, and 492 mμ lines were used for excitation. However, the 436-mμ line resulted in an additional fluorescent shoulder at 475 mμ. 2-Methoxyestrone (10 μg in 80% (v/v) H_2SO_4) was irradiated with light of 313, 365, 405, and 436 mμ. Figure 1 shows that incident light of 436 mμ is the most effective in

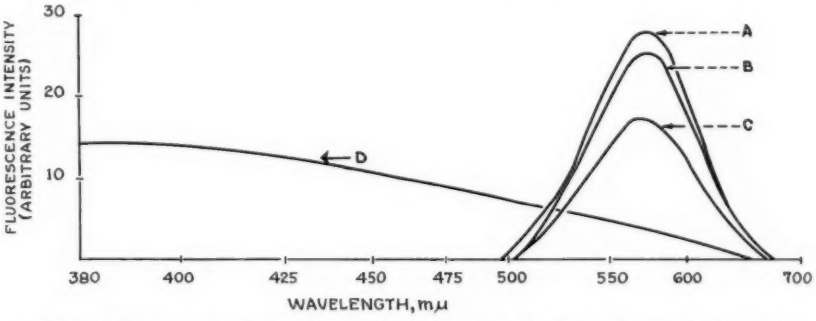


FIG. 1. Fluorescence spectra of 2-methoxyestrone. Exciting wavelengths: 436 mμ (A), 405 mμ (B), 365 mμ (C), 313 mμ (D).

producing fluorescence. A fluorescence peak at 579 mμ appears on excitation with light of 365, 405, and 436 mμ. However, a broad fluorescence spectrum ranging from 340 to over 600 mμ is recorded when the 2-methoxyestrone solution is irradiated with light of 313 mμ.

Effect of Heating Time on Estrogen Fluorescence

Estradiol-17β (0.4 μg), estrone (0.4 μg), 16α-hydroxyestrone (0.6 μg), estriol (0.4 μg), 16-epiestriol (0.4 μg), and 2-methoxyestrone (0.4 μg) in 75%

(v/v) H_2SO_4 were heated in a boiling-water bath for 5, 10, 15, 20, and 30 minutes. Figure 2 shows the individual values of the quadruplicate analyses at each

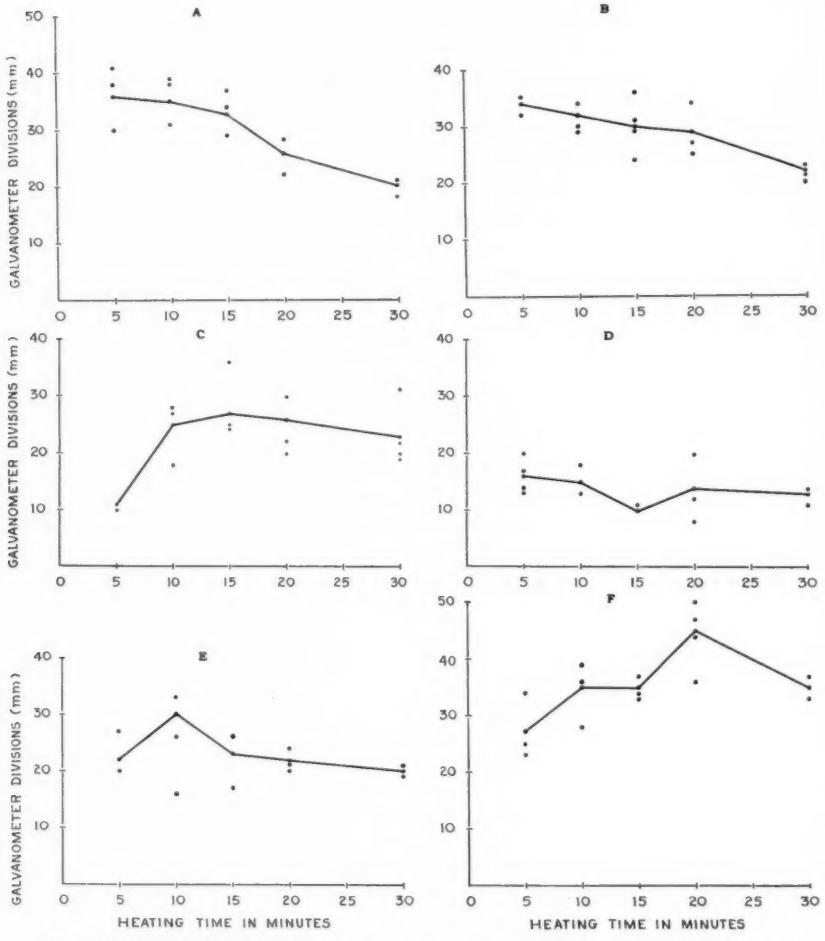


FIG. 2. Effect of the duration of heating on the fluorescence of estradiol-17β (A), estrone (B), estriol (C), 16-epiestriol, (D), 16α-hydroxyestrone (E), and 2-methoxyestrone (F).

heating time. The mean values are joined by a line. Estradiol-17β, estrone, and 16-epiestriol require only 5 minutes of heating for maximum fluorescence intensity. 16α-Hydroxyestrone, estriol, and 2-methoxyestrone require 10, 15, and 20 minutes of heating respectively. Figures 2A and 2E suggest that a 30-minute heating time results in better reproducibility for estradiol-17β and 16α-hydroxyestrone.

Relative Fluorescence Intensity at Room Temperature

Ten micrograms of estradiol-17β, estrone, 16α-hydroxyestrone, and estriol were left standing in concentrated sulphuric acid at room temperature for 10 minutes, and their fluorescence spectra determined. The relative fluorescence intensities were 67 (estradiol-17β), 23 (estrone), 8 (16α-hydroxyestrone), and 0 (estriol) when compared with the heated estrone standard described above.

Influence of Acid Concentration and the Addition of Ethanol or Toluene–Ethanol on the Fluorescence and Absorption Spectra

(a) Estrone.—The relative fluorescence intensity and absorbance of estrone change with acid concentration (Figs. 3A and 3B). Figure 3B illustrates that

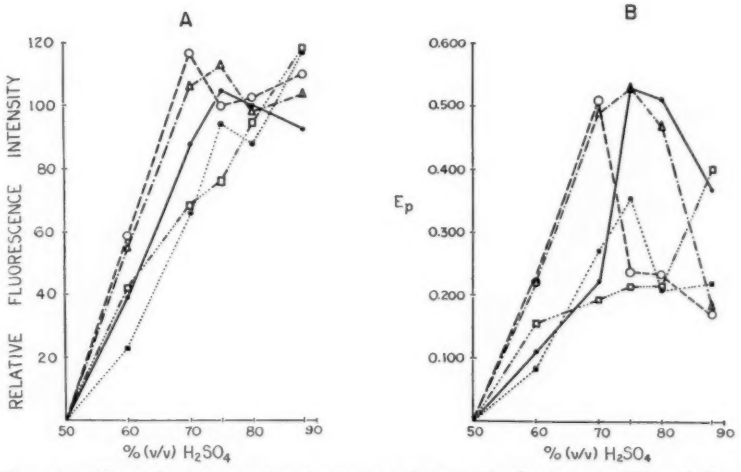


FIG. 3. Effect of acid and ethanol concentrations on the fluorescence (A) and absorbance (B) of estrone. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

the solutions develop more color as more water is added. The wavelength of peak absorption also changes with acid concentration. The shoulder of the absorption curve gradually disappears with increasing acid concentration. Fluorescence intensity increases as water content is increased, but falls off sharply above 30% water. The wavelength of maximum fluorescence changes with acid concentration (Fig. 11A). Ethanol appears to enhance fluorescence. The addition of toluene–ethanol to an aqueous sulphuric acid solution of estrone increases the absorbance by 22% and the fluorescence intensity by 18% (Table III and Fig. 13).

(b) Estradiol-17β (Figs. 4 and 14).—As the acid concentration is increased from 75 to 88% there are significant changes in the absorption and fluorescence spectra. With 75% H_2SO_4, the wavelength of peak absorption is at 455 mμ, whereas with 80% H_2SO_4, an absorption shoulder appears at 433 mμ. This shoulder changes to a peak at 428 mμ when the acid concentration is increased

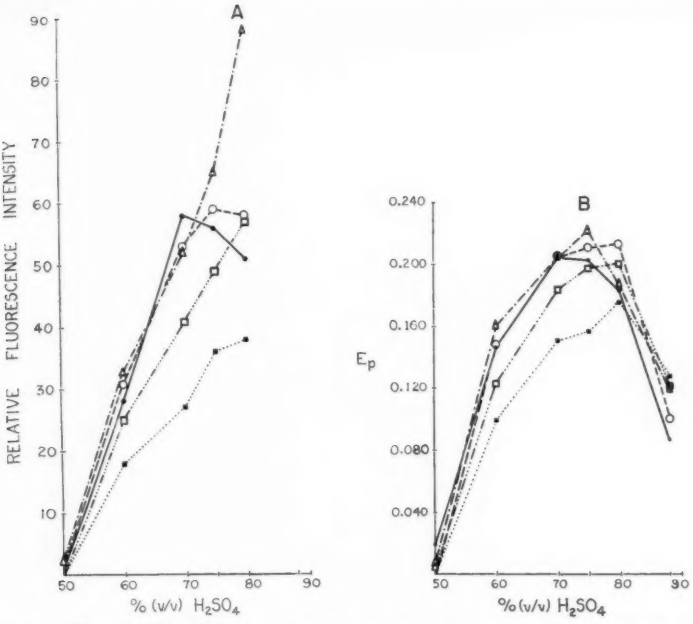


FIG. 4. Effect of acid and ethanol concentrations on the fluorescence (A) and absorbance (B) of estradiol-17β. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

to 88%. The peak of the fluorescence spectrum is at 482 mμ with 75% acid. A shoulder at 447 mμ appears with 80% acid, and it becomes the major fluorescence peak at 448 mμ with 88% acid. Ethanol does not appear to have much effect except in 80% acid and 0.1 ml ethanol values. The addition of toluene–ethanol increases the absorbance by 11% and the fluorescence by 17% (Table III and Fig. 14).

(c) Estriol.—The addition of 0.05 ml ethanol to estriol in solutions of 70, 75, and 80% H_2SO_4 results in a very significant increase in fluorescence inten-

TABLE I

The relative fluorescence intensities* of eight natural estrogens under optimal acid and ethanol concentrations

Estrogen	R.F.I.*	Peak, mμ	H_2SO_4, %(v/v)	Ethanol, ml
16-Epiestriol	139	479	88	0.40
Estradiol-17β	117	470	88	0.10
Estriol	105	480	75	0.05
Estrone	100	478	88	0.20
16-Ketoestradiol-17β	26	528	80	0
16α-Hydroxyestrone	14	527–529	75	0
16-Ketoestrone	9	518	60	0
2-Methoxyestrone	1	579	50	0

*The relative fluorescence intensity of an estrogen solution when compared to 11.2 μg estrone in 3 ml 80% (v/v) H_2SO_4 rendered fluorescent by heating 10 minutes in a boiling-water bath. The R.F.I.'s are corrected for differences in estrogen concentration.

sity (Fig. 5A). This increase in fluorescence cannot be explained by the dilution of H_2SO_4 by ethanol (Table II). The estriol absorption spectrum in 60% H_2SO_4 appears as flat shoulders at 448 and 510 mμ. The shoulder at 448 mμ becomes a peak at higher acid concentrations. Figure 5B shows that the addition of 0.05 ml ethanol to 70, 75, and 80% acid solutions of estriol increases the

TABLE II

Dilution of sulphuric acid by ethanol

Ethanol (ml) added to 3 ml of:	0	0.05	0.1	0.2	0.4
% (v/v) H_2SO_4	50	—	—	—	—
	60	59.0	58.1	56.3	52.9
	70	68.9	67.7	65.6	61.8
	75	73.8	72.6	70.3	66.2
	80	78.7	77.4	75.0	70.6
	88	86.6	85.2	82.5	77.6

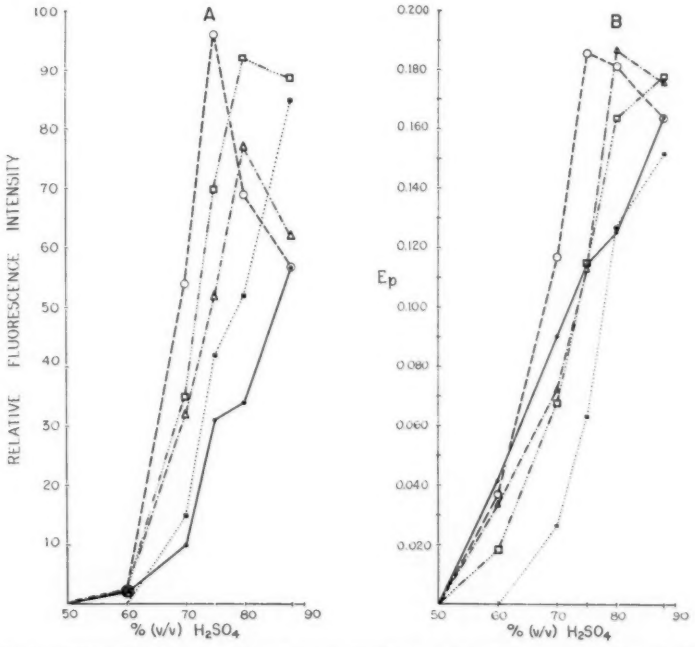


FIG. 5. Effect of acid and ethanol concentrations on the fluorescence (A) and absorbance (B) of estriol. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

absorbance. Figure 12A illustrates that a change in the acid concentration from 70% to 88% results in a shift of the fluorescence peak from 482 to 475 mμ. The addition of toluene–ethanol results in an increase in fluorescence (Table III) which is illustrated in Fig. 15. In this experiment, the increase in fluorescence

TABLE III

The effects of toluene–ethanol (19:1) on the fluorescence and absorption spectra of eight natural estrogens

	Absorption with 75% (v/v) H_2SO_4		Absorption with 75% (v/v) H_2SO_4 plus toluene–ethanol			Fluorescence with 75% (v/v) H_2SO_4			Fluorescence with 75% (v/v) H_2SO_4 plus toluene–ethanol		
Estrogen, μg	Absorbance	Peak, mμ	Absorbance	Peak, mμ	% increase with toluene–ethanol	Intensity*	R.F.I.†	Peak, mμ	Intensity*	Peak, mμ	Δ intensity(%)‡ with toluene–ethanol
Estrone (10.2)	0.36		0.43			22			26		
	0.36	455	0.44	455	22	21	100	484	26	484	+18
16-Epiestriol (9.2)	0.19		0.27			16			20		
	0.20	454	0.28	454	40	17	79	480	21	483	+24
Estradiol-17β (10.0)	0.19		0.21			12			13		
	0.19	456	0.21	456	11	12	68	482	14	483	+17
Estriol (10.0)	0.10		0.20			6			15		
	0.11	453	0.20	453	82	6	32	479	15	481	+150
16-Ketoestradiol-17β (10.0)	0.17		0.24			6			3		
	0.17	484	0.24	502	41	6	29	528	3	533	−50
16α-Hydroxyestrone (11.9)	0.15		0.18			3			3		
	0.15	503	0.18	504	20	3	6	531	3	533	0
16-Ketoestrone (10.0)	0.02		0.02		Insufficient	3			8		
	0.02	450	0.03	450	accuracy	3	4	515	7	515	+166
2-Methoxyestrone (9.8)	0.12		0.12		Insufficient	2			2		
	0.12	487	0.13	487	accuracy	2	2	579	2	579	0

*Arbitrary units.

†R.F.I. is the relative fluorescence intensity of an estrogen solution when compared with the intensity of 10.2 μg estrone in 3 ml 75% (v/v) H_2SO_4. The values of the estrone reference standard used in the calculations of the R.F.I. are not shown. The R.F.I.'s are corrected for differences in estrogen concentration.

‡In another series of experiments under similar conditions, the results were the same with all the estrogens except 2-methoxyestrone which had a Δ intensity of +6%.

is 150%. Moreover, quantitative studies with the Farrand instrument suggest that the effect cannot be attributed to dilution, since a lower acid concentration or the substitution of toluene–ethanol by ethanol does not produce the same response (Table IV). Toluene–ethanol increases the absorbance of estriol at its peak wavelength by 82%, but decreases it at the shoulder (Table III). Similar effects on the shoulder diminution were noted with 16-epiestriol, estrone, and estradiol-17β.

TABLE IV

The effect of ethanol and toluene–ethanol (19:1) on estriol fluorescence

Expt. No.	Solvent added, ml	% (v/v) H_2SO_4	R.F.I. of solution		
			Blank		Estrogen
1	0	70	8.0		13.3 13.5 13.9 17.0
				Mean	14.4
2	0	75	9.0		27.9 25.9 26.1 29.0
				Mean	27.2
3	Ethanol (0.2)	75	8.2		23.2 20.0 26.0 23.0
				Mean	23.1
4	Toluene–ethanol (0.2)	75	10.0		57.0 57.0 53.5 55.5
				Mean	55.6
5	Toluene–ethanol (0.1)	75	9.0		55.3 55.3 55.3 55.0
				Mean	55.2

(d) 16-Epiestriol.—The addition of ethanol to an acid solution of 16-epiestriol, in contrast to its addition to estriol, does not have a pronounced effect on fluorescence. Moreover, the optimum acid and ethanol concentrations for maximum fluorescence are different for these epimers (Table I). Figure 6B illustrates the absorbance of 16-epiestriol in different acid concentrations. With 60% H_2SO_4, the absorption spectrum of 16-epiestriol shows a peak at 458 mμ and a shoulder at 510 mμ, whereas estriol shows spectral shoulders at 448 and 510 mμ. Figure 12B illustrates the shift in the fluorescence peak of 16-epiestriol from 487 to 474 mμ when the acid concentration is changed from 60 to 88% H_2SO_4. This shift is very marked in the change from 80 to 88%. Moreover, with increasing sulphuric acid concentration the difference between the wavelength of maximum absorption and the wavelength of maximum emission becomes smaller. The addition of toluene–ethanol increases the fluorescence

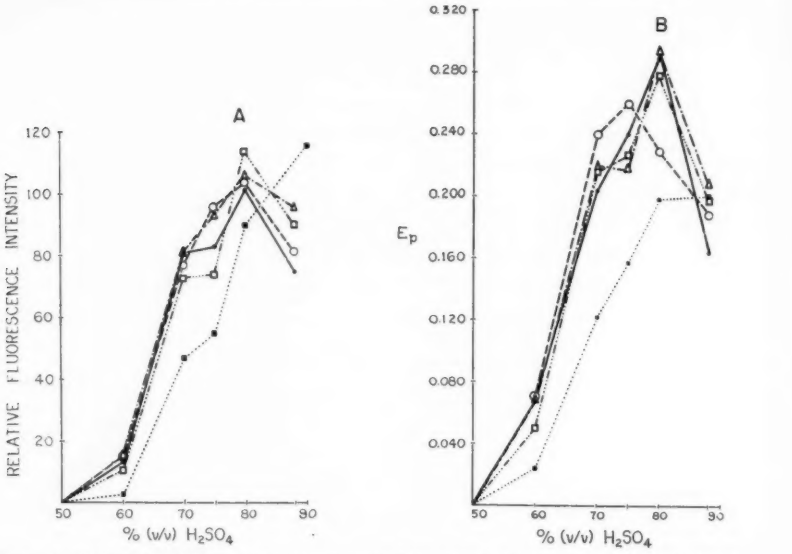


FIG. 6. Effect of acid and ethanol concentration on the fluorescence (A) and absorbance (B) of 16-epiestriol. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

by 24% and shifts the fluorescence peak from 480 to 483 mμ (Table III and Fig. 16). Thus, the toluene–ethanol effect is much less pronounced with 16-epiestriol than with estriol.

(e) 16-Ketoestradiol-17β.—The addition of ethanol diminishes fluorescence (Fig. 7A) and produces a shift in the absorption peak. Figure 7B illustrates the changes in absorbance with acid concentration. When the acid concentration is changed from 60 to 88% there is a shift in the fluorescence peak from 535 to 528 respectively (Fig. 11B) and the absorption peak is shifted from 488 to 495 mμ. When toluene–ethanol is added, the absorption peak is shifted from 484 to 502 mμ, and the fluorescence peak from 528 to 533 mμ (Table III and Figs. 17, 19). Of the eight estrogens studied, 16-ketoestradiol-17β showed the most striking spectral changes on the addition of toluene–ethanol, a 50% decrease in fluorescence, a 41% increase in absorbance, and the most pronounced shift in the position of the two maxima.

(f) 16α-Hydroxyestrone.—The addition of ethanol decreases fluorescence (Fig. 8A). Figure 8B shows the effect of acid concentration on absorbance. The absorption peak of 16α-hydroxyestrone was shifted from 490 mμ in 100% acid to 505 mμ in 70% acid. The optimum acid concentration for the fluorescence of 16α-hydroxyestrone is 75% while that for 16-ketoestradiol-17β is 80% (Table I). The addition of toluene–ethanol to a 16α-hydroxyestrone solution does not shift the absorption peak (Table III and Fig. 18), does not affect the fluorescence intensity, and increases the absorbance only 20% (*vide supra*).

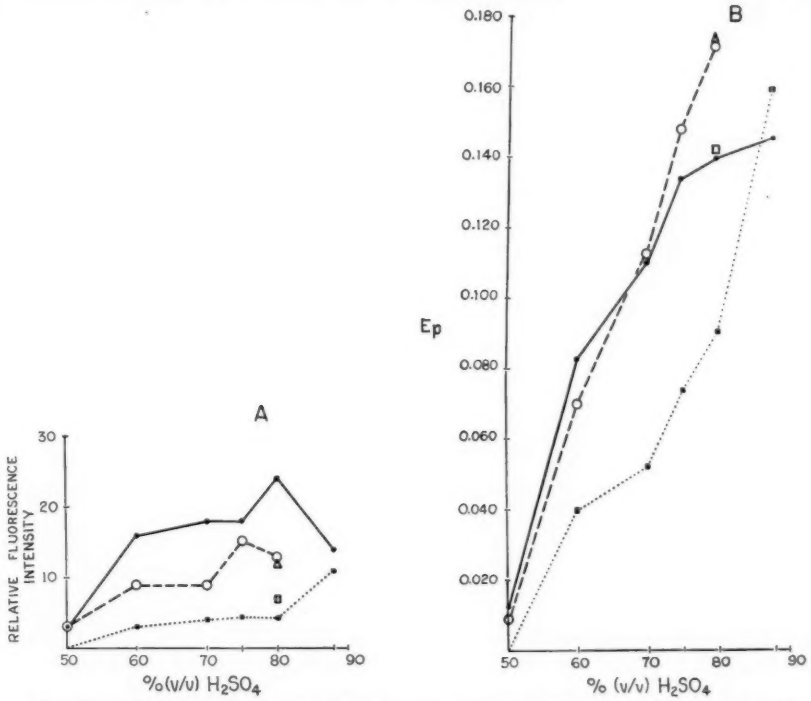


FIG. 7. Effect of acid and ethanol concentrations on the fluorescence (A) and absorbance (B) of 16-ketoestradiol-17β. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

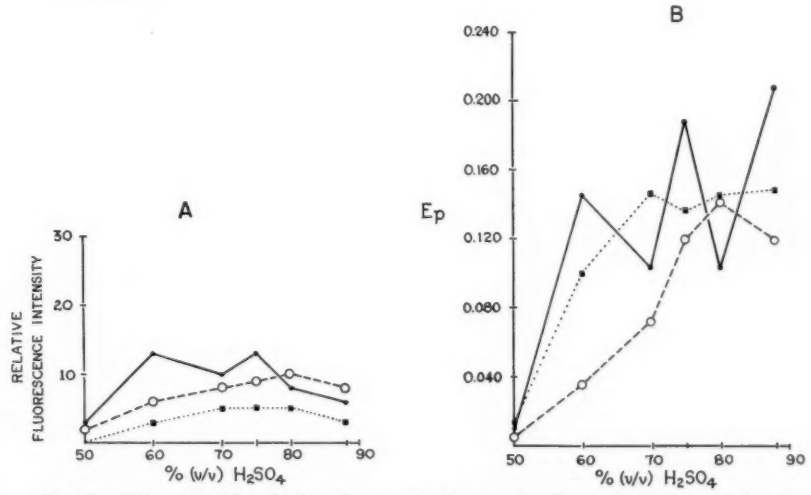


FIG. 8. Effect of acid and ethanol concentrations on the fluorescence (A) and absorbance (B) of 15α-hydroxyestrone. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

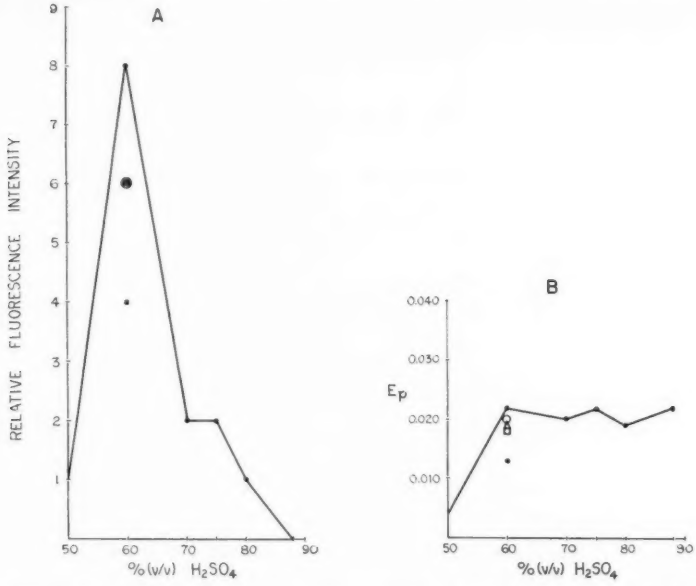


FIG. 9. Effect of acid and ethanol concentrations on the fluorescence (A) and absorbance (B) of 16-ketoestrone. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

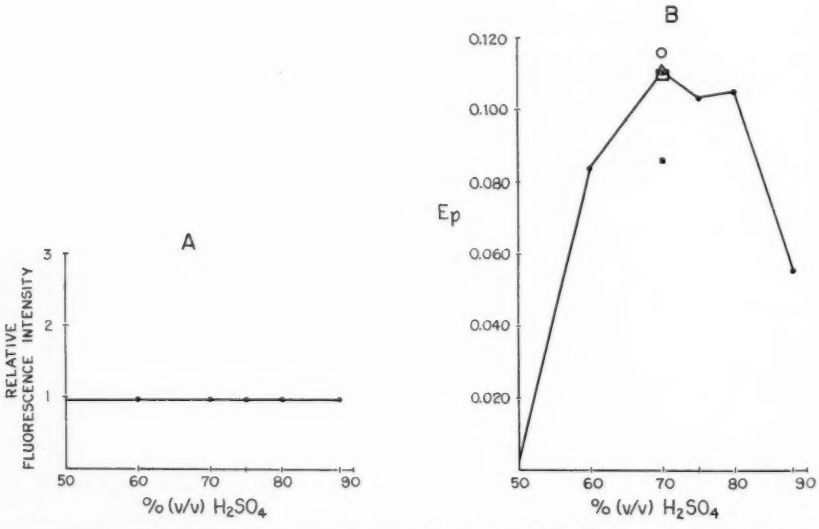


FIG. 10. Effect of acid and ethanol concentrations and the fluorescence (A) and absorbance (B) of 2-methoxyestrone. Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

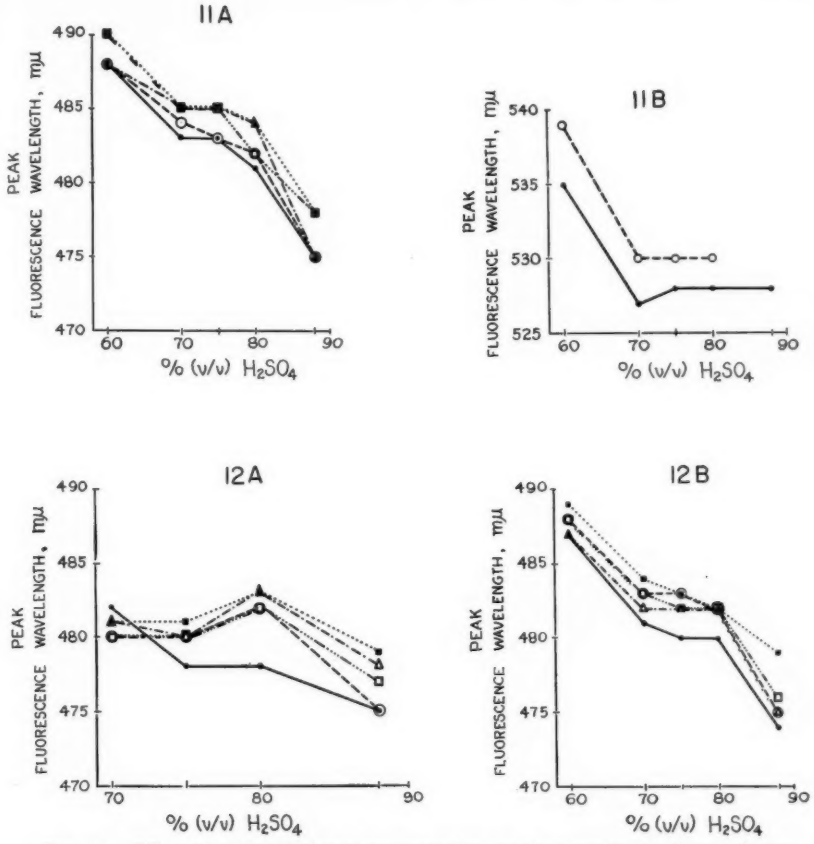
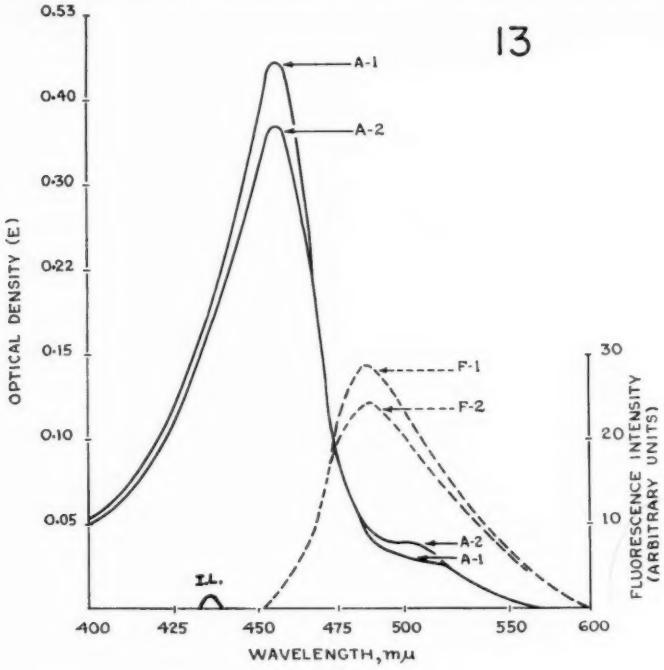


FIG. 11. Effect of acid and ethanol concentrations on the wavelength of peak fluorescence of estrone (A) and 16-ketoestradiol-17β (B). Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

FIG. 12. Effect of acid and ethanol concentrations on the wavelength of peak fluorescence of estriol (A) and 16-epiestriol (B). Ethanol addition (ml): ● = zero, ○ = 0.05, △ = 0.1, □ = 0.2, ■ = 0.4.

(g) 16-Ketoestrone.—The fluorescence and absorbance in sulphuric acid are low (Fig. 9); the only distinct fluorescence spectrum is with 60% acid. At 60% acid, there is an absorption maximum at 458 mμ. At other acid concentrations there is a broad absorption shoulder from 400 to 600 mμ characterized by a slight rise at 458 mμ. Table III and Fig. 20 show that toluene–ethanol increases the fluorescence intensity.

(h) 2-Methoxyestrone.—Figure 10 shows that despite the high absorbance, the fluorescence yield is low. The addition of toluene–ethanol has little or no effect on the fluorescence intensity. 2-Methoxyestrone shows a fluorescence peak at 579 mμ (Fig. 21).



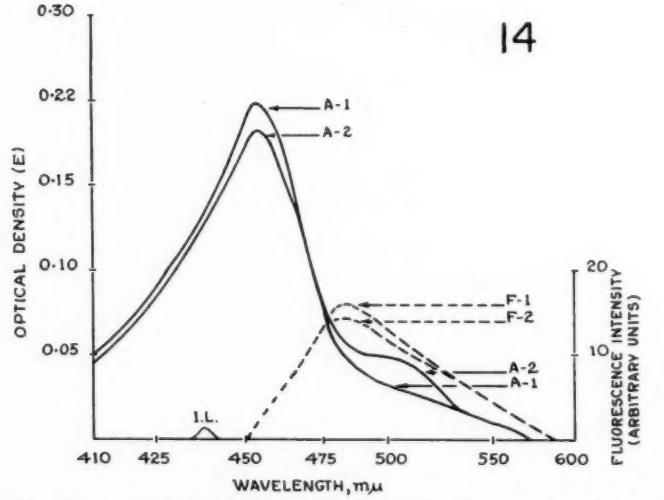
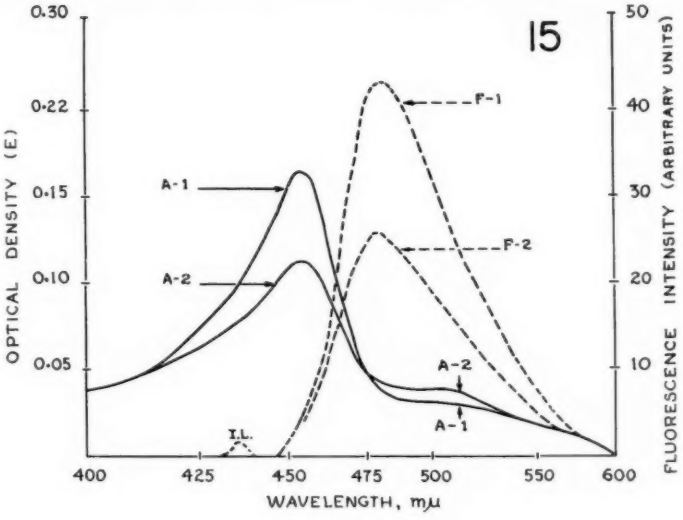


FIG. 13. Absorption (A) and fluorescence (F) spectra of estrone in 75% (v/v) H_2SO_4 with (A-1, F-1) and without (A-2, F-2) the addition of toluene–ethanol. Incident light = I.L.

FIG. 14. Absorption (A) and fluorescence (F) spectra of estradiol-17β in 75% (v/v) H_2SO_4 with (A-1, F-1) and without (A-2, F-2) the addition of toluene–ethanol. Incident light = I.L.



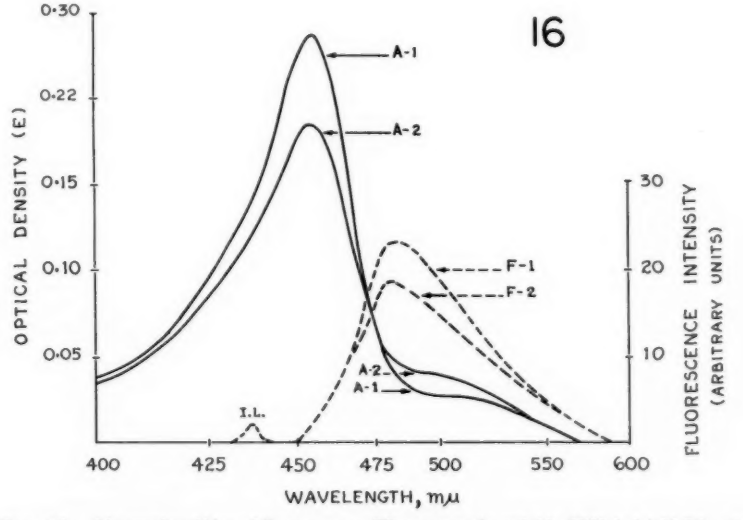


FIG. 15. Absorption (A) and fluorescence (F) spectra of estriol in 75% (v/v) H_2SO_4 with (A-1, F-1) and without (A-2, F-2) the addition of toluene–ethanol. Incident light = I.L.

FIG. 16. Absorption (A) and fluorescence (F) spectra of 16-epiestriol in 75% (v/v) H_2SO_4 with (A-1, F-1) and without (A-2, F-2) the addition of toluene–ethanol. Incident light = I.L.

Relative Fluorescence under Optimum Conditions

Table I lists the relative fluorescence intensities of eight natural estrogens under optimum acid and ethanol concentrations. The R.F.I. values are corrected for small differences in estrogen concentrations at the time of the studies. 16-Epiestriol under these conditions is the most and 2-methoxyestrone the least fluorogenic. Table III lists the relative fluorescence intensities under similar acid concentrations.

Fluorescence and Absorption Spectra

Figures 13 to 21 illustrate the fluorescence and absorption spectra of eight natural estrogens under the same acid conditions (75% H_2SO_4) with and without the addition of toluene–ethanol (0.1 ml). The results are summarized in Table III.

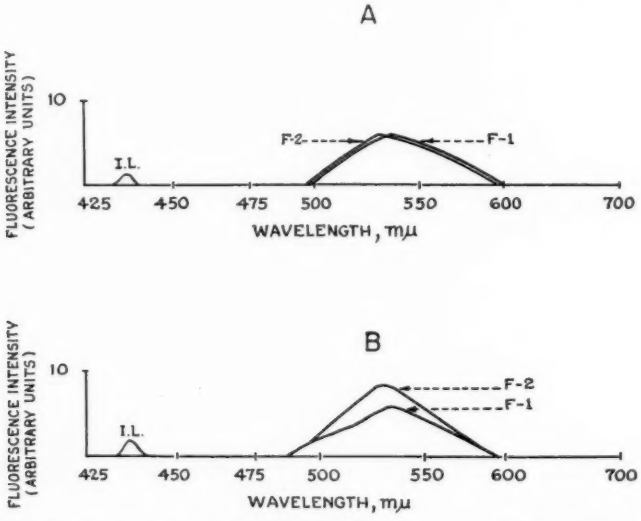
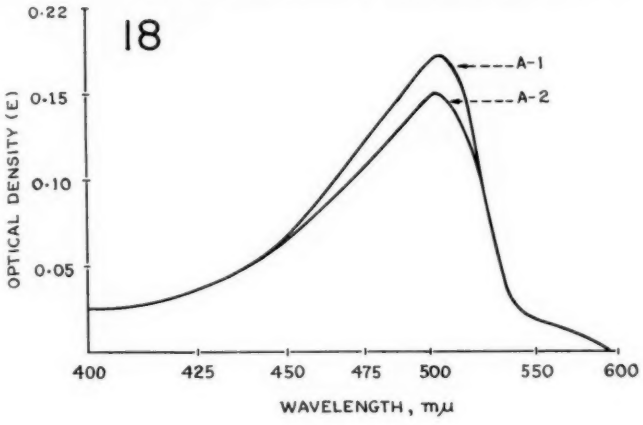


FIG. 17. Fluorescence spectra of 16α-hydroxyestrone (A) and 16-ketoestradiol-17β (B) in 75% (v/v) H_2SO_4 with (F-1) and without (F-2) the addition of toluene–ethanol. Incident light = I.L.



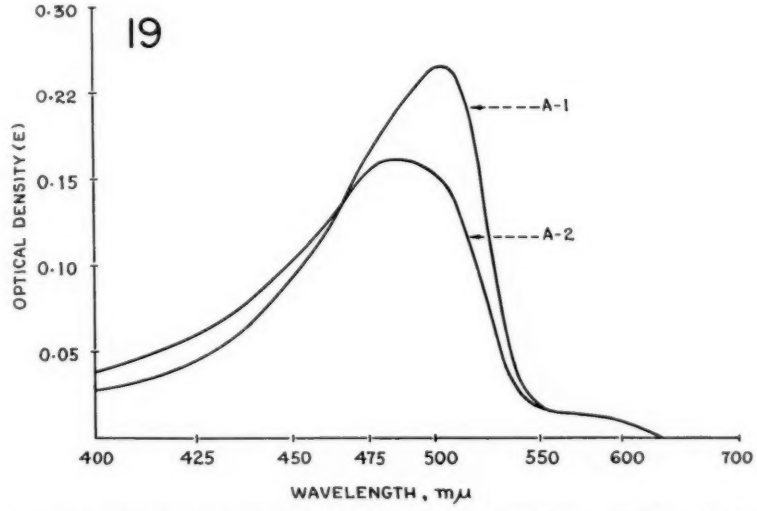


FIG. 18. Absorption spectra of 16α-hydroxyestrone in 75% (v/v) H_2SO_4 with (A-1) and without (A-2) the addition of toluene–ethanol.

FIG. 19. Absorption spectra of 16-ketoestradiol-17β in 75% (v/v) H_2SO_4 with (A-1) and without (A-2) the addition of toluene–ethanol.

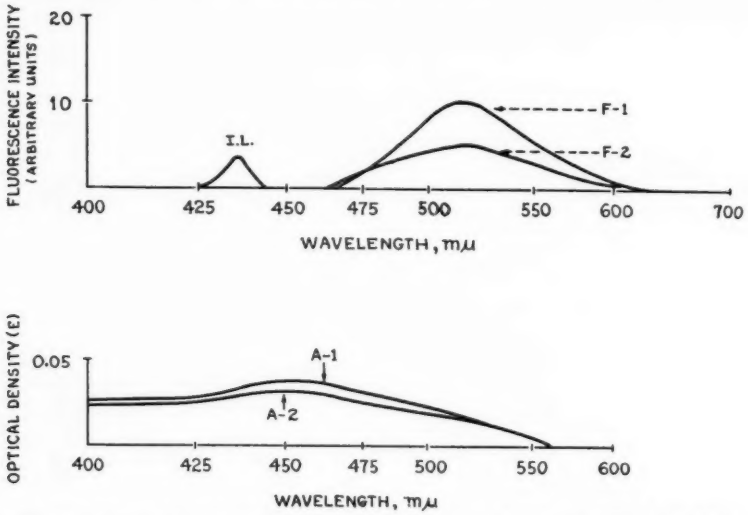


FIG. 20. Absorption (A) and fluorescence (F) spectra of 16-ketoestrone in 75% (v/v) H_2SO_4 with (A-1, F-1) and without (A-2, F-2) the addition of toluene–ethanol. Incident light = I.L.

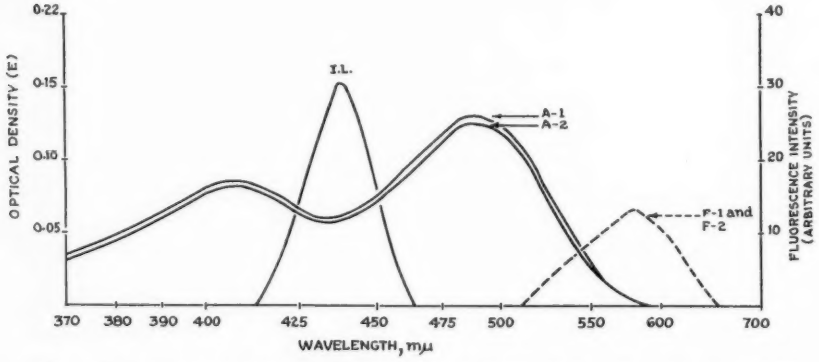


FIG. 21. Absorption (A) and fluorescence (F) spectra of 2-methoxyestrone in 75% (v/v) H_2SO_4 with (A-1, F-1) and without (A-2, F-2) the addition of toluene–ethanol. Incident light = I.L.

Discussion

The foregoing experiments demonstrate how the fluorescence intensities and the fluorescence spectra of eight natural estrogens are affected by incident light, time of heating, sulphuric acid concentration, and the addition of ethanol or toluene–ethanol. Similarly, the absorbances and absorption spectra are affected by acid concentration and toluene–ethanol.

The observations on the effect of incident light on the fluorescence spectra of the three classical estrogens confirm the work of Aitken and Preedy (1) and Goldzieher (15). Moreover, the experiments show that the 16α-hydroxyestrone

and 2-methoxyestrone fluorescence spectra change qualitatively with specific bands of incident light. The broad fluorescence spectrum of 2-methoxyestrone which is emitted by incident light at 313 mμ is of particular interest.

In a study on heating times required for maximum fluorescence, Diczfalusy (9) found that 20 minutes are required for estriol and 10 minutes for estradiol-17β. Brown (7) observed that estrone and estradiol-17β reached their maximum fluorescence in 5 minutes, while estriol fluorescence was still increasing after 20 minutes. Although a just comparison cannot be made because of the different conditions, it is of interest that the present results on estrone are in agreement with the work of Brown. Furthermore, the two isomers, 16-epiestriol and estriol, require different heating times for maximum fluorescence. Marrian and Bauld (17) reported that 16-epiestriol required a much shorter time for the first stage of the Kober reaction than estriol, and suggested that the *cis*-configuration of 16-epiestriol is more sensitive to dehydration by sulphuric acid. Furthermore, the results of the fluorescence experiments at room temperature indicate the lower reactivity of estriol when compared with estradiol-17β, estrone, and 16α-hydroxyestrone under certain conditions.

The acid concentration is important for maximum fluorescence, absorbance, and spectral specificity. Thus, the fluorescence and absorption peaks of estradiol-17β change with varying acid concentration. This phenomenon was noted earlier by Linford (16) and by Umberger and Curtis (21). There is also a gradual shift of the absorption peak of 16α-hydroxyestrone with a change in acid concentration. In concentrated sulphuric acid, the peak is similar to that of 16-ketoestradiol-17β, but changes to one of longer wavelength as the acid becomes more dilute. With estrone, estriol, 16-epiestriol, and 16-ketoestradiol-17β, a gradual increase in acid concentration results in a reduction in the difference between the wavelength of maximum absorption and the wavelength of maximum emission.

The enhancement of the fluorescence and absorption of estriol does not seem to be related in any simple way to the amount of ethanol added, nor does the dilution of sulphuric acid appear to be the explanation. Bates (2) has ascribed the effect of ethanol to dilution. Brown (8) has suggested that estriol requires the protection of reducing agents (for example, ethanol) to ensure maximum production of fluorescence. However, it should be noted that 16-epiestriol, estradiol-17β, 2-methoxyestrone, 16-ketoestrone, and 16α-hydroxyestrone do not show increased fluorescence with ethanol. In fact, 16-ketoestradiol-17β becomes less fluorogenic under these conditions.

The addition of toluene–ethanol results in increased absorbance and fluorescence of some of the estrogens. The steroid specificity of toluene–ethanol is demonstrated again by its unusual effect on the absorption spectrum of 16-ketoestradiol-17β and its lack of a qualitative effect on the isomeric 16α-hydroxyestrone. These findings were unexpected, since previous workers (3, 10, 13) used toluene–ethanol as a solvent for the steroid samples. Engel *et al.* (10, 20) found that toluene itself gives higher readings and that the spectra of estrone, estradiol-17β, and estriol were the same with and without toluene–ethanol. Recently Epstein *et al.* (11) reported that the addition of *p*-toluene-

sulphonic acid crystals to steroids will result in high fluorescence under certain conditions. Therefore, the addition of toluene–ethanol to sulphuric acid solutions may result in the formation of toluenesulphonic acid, and it may be this type of compound which shows specific reactivity with the estrogens. This phenomenon may be of value in increasing the sensitivity of fluorometric measurements and for the differential estimation of 16α-hydroxyestrone and 16-ketoestradiol-17β.

The fluorescence spectra of the three classical estrogens are in agreement with the work of others (1, 15, 18, 20). The fluorescence spectrum of 16-ketoestradiol-17β reported by Bompiani (5) has a peak at a longer wavelength; however, the experimental conditions were different in the two cases. Slaunwhite *et al.* (19) reported the fluorescence intensity of 16-ketoestradiol-17β and 16-ketoestrone. Breuer and Knuppen (6) recently reported absorption peaks of 16α-hydroxyestrone and 16-epiestriol in aqueous sulphuric acid which are in agreement with an earlier report (12). 2-Methoxyestrone has very minor and qualitatively characteristic fluorogenic properties which may be valuable in differential fluorometry. Furthermore, the distinctive fluorescence characteristics of estradiol-17β, 16α-hydroxyestrone, 16-ketoestradiol-17β, 16-ketoestrone, and 2-methoxyestrone emphasize the fluorescence specificity of the phenolic A-ring compounds.

Acknowledgments

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RECORDING SPECTROFLUOROMETERS: TWO NEW DESIGNS AND THEIR EVALUATION[1]

JOSEPH W. GOLDZIEHER,[2] WILLIAM S. BAULD,[3] LEWIS L. ENGEL,[4] AND MORRIS L. GIVNER[5]

Abstract

The expanding importance of fluorometry as an analytical tool necessitated an investigation of instrumental design and theory as applied to high-speed recording instruments. The construction and performance of two new spectrofluorometers are described and compared with a commercially available instrument.

Introduction

The development of fluorometric methods has often been impeded by the lack of instruments for the examination of the entire fluorescence spectrum of the test material. In the choice of optimum conditions for fluorometry, knowledge of the fluorescence spectra of test material and possible contaminants is of prime importance just as is knowledge of the absorption spectra of test material and contaminants in colorimetry. Efforts have been made to solve this problem with spectrographic methods (12), but these introduce all the difficulties attendant upon the use of photographic emulsions. Others have made approximations of the fluorescence spectrum with a series of interference filters (3). To achieve more than a first approximation of the fluorescence spectrum by this means is a formidable mathematical task (8, 10). Far more precise, but also extremely tedious, is the point by point measurement of the fluorescence spectrum, the method which has been used in most investigations to date (10, 16, 1).

The extreme sensitivity of fluorometric methods for the measurement of estrogens has aroused wide interest among workers in this field and a number of procedures have been described, differing in the reagents used, the development time, and in a variety of other parameters (2, 6, 7, 11, 16). At a conference in

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Contribution from the Department of Endocrinology, The Southwest Foundation for Research and Education, San Antonio, Texas; the Department of Metabolism, The Montreal General Hospital, Montreal, Que.; and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts.

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[2]Department of Endocrinology, The Southwest Foundation for Research and Education, San Antonio, Texas.

[3]Department of Metabolism, The Montreal General Hospital, Montreal, Que. Dr. Bauld died July 20, 1958.

[4]Permanent Faculty Fellow of the American Cancer Society, The John Collins Warren Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University at the Massachusetts General Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts. The portion of the work done in these laboratories was supported by grants from the National Cancer Institute, United States Public Health Service, a grant from the American Cancer Society, Inc., and a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

[5]Department of Metabolism and the McGill University Clinic, The Montreal General Hospital, Montreal, Que.; Fellow of the Salmon Foundation for Medical Research, Inc.; work supported in part by grants from National Research Council of Canada, Maurice Pollack Foundation, and David L. Harrington, Esq. Present address: Department of Endocrinology, The Southwest Foundation for Research and Education, San Antonio, Texas.

1956 (13) this subject was discussed exhaustively and it appeared that a detailed analysis of these factors would delineate optimum conditions of measurement for the classical as well as the newly discovered estrogens.

Faced with this array of methods and individual variables it was obvious that a high speed recording spectrofluorometer would be required to accomplish the task. This spurred investigators into the design and construction of such equipment. The basic features of such instruments are shown in Fig. 1.

The light from the mercury–xenon arc (1 in Fig. 1) is focused on the entrance slit of the primary monochromator (2 in Fig. 1). The incident light emerges from this monochromator in a high degree of spectral purity and is focused on the sample cuvette (3 in Fig. 1) containing the estrogen rendered fluorescent by previous heating in aqueous sulphuric acid. The emitted fluorescence from the sample cell is focused on the entrance slit of a secondary monochromator (4 in Fig. 1). This light then passes to a photomultiplier (5 in Fig. 1) and recorder (6 in Fig. 1).

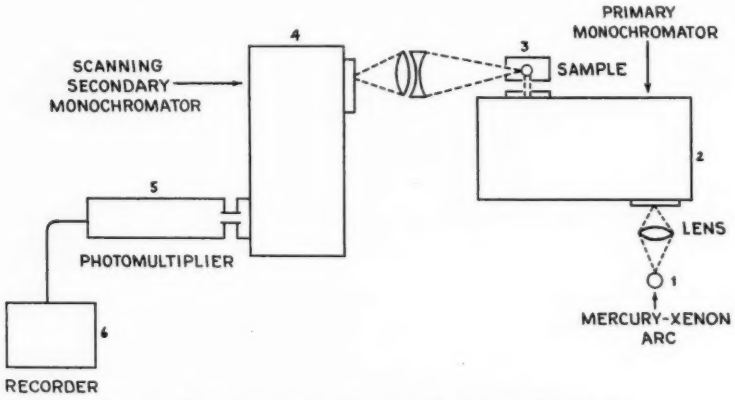

FIG. 1. Basic components of a recording spectrofluorometer.

Spectrofluorometers present certain unique problems: two types of radiation (exciting and fluorescent) must be considered individually and must be separated sharply, since the intensity of the exciting radiation is so much greater than the fluorescence output. For this reason, particular attention must be given to the *extreme* bandwidth of the radiation emerging from the excitation monochromator. In fluorometry the limiting instrumental factors are the available excitation energy and the sensitivity of the detector. From the point of view of instrumental design, extreme sensitivity with adequate resolution is required of a spectrofluorometer. In the course of experimentation it also became apparent that a wide range of undistorted signal amplification was an additional desirable feature.

Accordingly, two recording instruments were developed, one based largely on Perkin–Elmer components, the other on the Beckman DK-2 recording spectrophotometer. Subsequent to the completion of these two instruments,

commercial spectrofluorometers became available, providing us with an opportunity of comparing the two new designs with an instrument manufactured by the American Instrument Company.

Materials

Instrument I

The fluorescence box is shown diagrammatically in Fig. 2. The light source consisted of a 1000-watt Hanovia xenon–mercury arc with accessory starting equipment. The lamp itself was housed in a protective metal cylinder 6 in. in

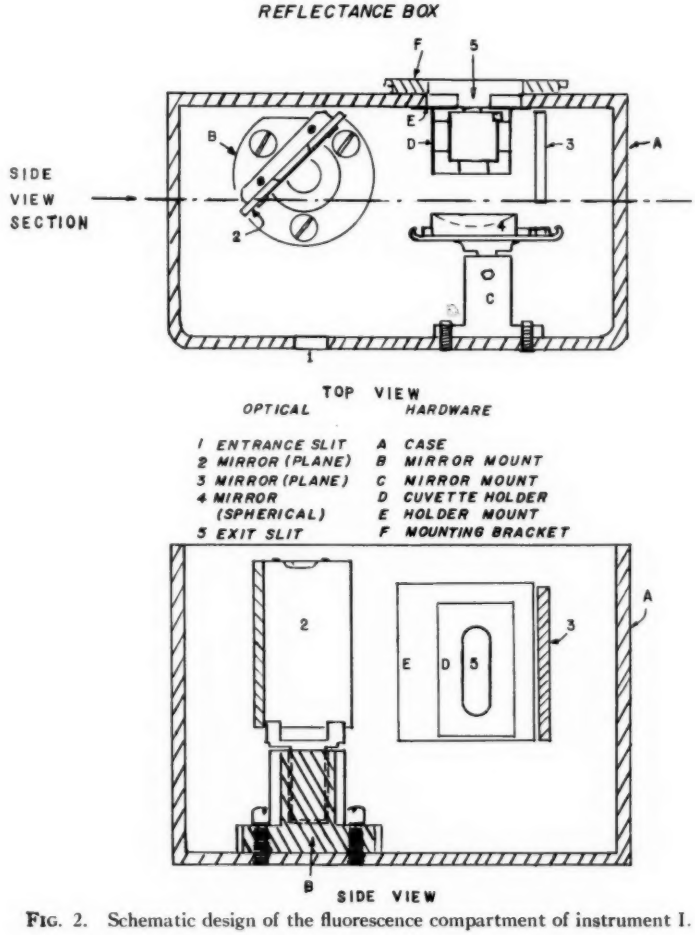

FIG. 2. Schematic design of the fluorescence compartment of instrument I.

diameter; tubing 1 in. in diameter directed an air blast from a small blower onto the stems of the bulb. The cable carrying the high-frequency starter current

to the lamp was insulated from the metal housing by a 2-in. bakelite disk fitted into the metal housing; the cable passed through the center of this disk. On a level with the lamp bulb, the metal housing had a tubular side-arm large enough in internal diameter to permit the collimating lens system of a Bausch and Lomb 250-mm monochromator (type 33–86–40) inserted therein in correct optical alignment. This instrument, with an aperture ratio of *f*/4.4 and fitted with a 50×50 mm grating, 1200 lines per mm, blazed for the ultraviolet, produced a dispersion of 33 Å/mm with a grating efficiency of 65% at 365 mm. The side wall of the monochromator facing the light source was protected from the heat of the lamp by a sheet of ¼-in. thick asbestos board covered with a sheet of aluminum foil. The emergent monochromatic radiation was focused through a B & L type 33–86–53 long-focus quartz lens onto the sample cuvette. A front-surfaced mirror set in a rotatable Perkin–Elmer No. 012–0037 mount greatly simplified focusing of the exciting radiation on the sample. The samples were contained in square silica cuvettes (Pyrocell Co.) identical in dimensions with the standard Beckman cuvette but polished on all four sides. The cuvette was inserted into a holder made by cutting off the end section of a standard Beckman No. 5010 cuvette carrier to the size shown. Slots were drilled into the two solid faces and the unit was soldered to the wall of the fluorescence box. Mirrors (3 and 4 in Fig. 2) were mounted as shown to increase the intensity of the exciting radiation impinging on the sample and to direct a greater amount of radiated fluorescence into the slit of the analyzing monochromator. For further efficiency, an inexpensive spectacle-quality quartz lens of 22 mm focal length was ground and inserted into the light path in front of the entrance slit of the analyzing monochromator. The two plane mirrors and the quartz lens increased the signal strength of the instrument by a factor of nearly five. The analyzing monochromator was a Perkin–Elmer type 99 double-pass instrument housing a 60×72 mm crystal quartz prism as the dispersing element. The instrument has an effective aperture of *f*/4.5 and the prism gives a dispersion of 25 Å/mm at 300 mμ, 50 Å/mm at 400 mμ, and 100 Å/mm at 500 mμ. A 1P21 photomultiplier specially selected for high sensitivity was used as a detector. Slit masks were positioned on the entrance and exit slits and an auxiliary black metal shield was mounted around the photomultiplier, leaving only a window large enough to encompass the beam emerging from the exit slit. This resulted in a substantial reduction of noise. The 13-cycle a-c. signal from the photomultiplier was amplified by a Perkin–Elmer Model 103 linear amplifier and fed to a Brown Electronik recorder. The standard Perkin–Elmer automatic wavelength drive and control were incorporated into the instrument. The only change made was in the wavelength marker pip, which was found to be annoyingly large at high amplification. The 0.22 μf capacitor C-501 was replaced by one of 0.1 μf. The high d-c. voltage required by the photomultiplier was provided by a Furst 710 P power supply. The recorder, electronic equipment, and standard Perkin–Elmer control panel were mounted on a commercial rack; the optical components were fastened by adjustable bolts to a board of laminated wood; and the blower for the lamp housing was mounted independently beneath the working surface. Flexible 1¼-in. air hose made of

spiral wire covered with plastic conducted the air blast from the blower to the air input of the lamp housing.

Instrument II

This instrument also used a 1000-watt Hanovia xenon–mercury arc, housed in metal and cooled by an air blast. The lamp was positioned directly in front of the collimating lens of a Bausch and Lomb 500-mm (type 33–86–45–49) monochromator employing a 100×100 mm grating with 1200 lines per mm. The aperture of this instrument is $f/4.4$; its grating efficiency is 65% at 265 mμ; and it has a dispersion of 16.5 Å per mm. The monochromatic exit beam was focused directly on the cuvette by the 33–86–53 quartz lens (without diaphragm) as indicated in Fig. 3. The cuvettes were the same type as those used

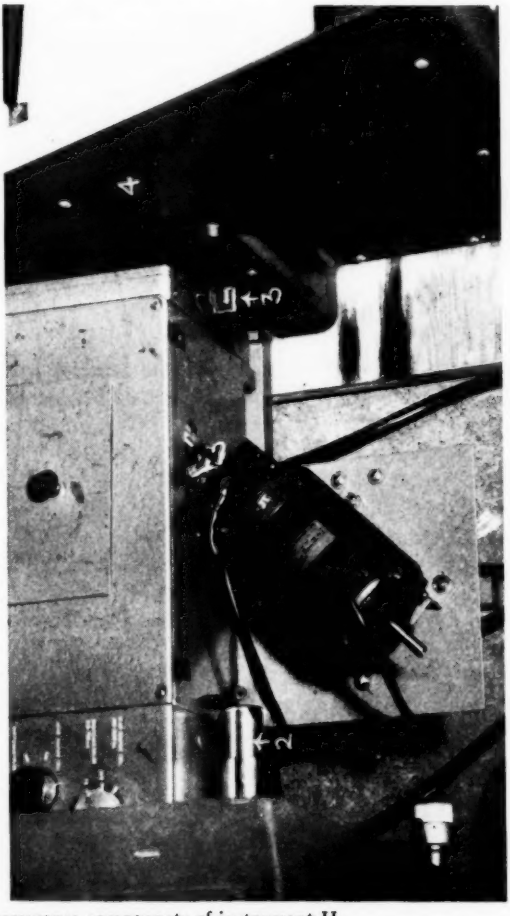

FIG. 3. Fluorescence components of instrument II.
(1) B and L primary monochromator, (2) exit lens of B and L, (3) fluorescence sample holder, (4) Beckman DK-2 spectrophotometer.

in instrument I, and were held in a sample holder as shown in Fig. 3. The fluorescent radiation travelled without collimation a distance of approximately 5 cm to the entrance mirror of a Beckman model DK-2 recording spectrophotometer. This instrument has a focal length of 500 mm, an effective aperture of *f*/10.4, and resolution characteristics of the DU quartz-prism instrument.

Instrument III

This instrument was the commercially available Aminco–Bowman spectrofluorometer. The light source consists of a 100-watt xenon arc with appropriate starter and transformers. The arc itself essentially forms the entrance slit, and the radiation is focused by a mirror onto a 50×50 mm grating ruled 600 lines/mm, providing a dispersion of 100 Å/mm. The radiation of selected wavelength passes through interchangeable fixed slits and impinges on a 1-cm silica cuvette similar to those used in the previously described instruments. The fluorescent radiation collected at right angles to the exciting beam is focused by a similar mirror and passes through a grating and slit system, finally reaching a 1P21 photomultiplier. The d-c. signal thus generated may be applied to a microphotometer, an oscilloscope, or a recorder. A motor drive is provided for both gratings so that a continuous excitation or fluorescence spectrum may be observed on a long-persistence oscilloscope.

Experimental

Instrumental Characteristics

Stability

Stability is defined as the measurement of the random fluctuations of the instrument during the determination of estrogen fluorescence intensities at the peak of the fluorescence spectrum. Instruments I, II, and III were tested after a minimum warm up period of 30 minutes.

Instrument I was tested with a solution of 2 μg/ml estrone in 80% sulphuric acid (v/v) heated at 80° C for 10 minutes. Excitation and analysis wavelengths were set for peak values (436 and 480 mμ respectively), gain at half of maximum (40 db), and slits were closed to give 80% of full-scale deflection. At this setting the instrument was operated continuously for 15 minutes. The recorded fluorescence varied a maximum of ±1% from the mean value. Instrument II was subjected to more rigorous testing. Estradiol-17β, 3.5 μg/ml in 75% (v/v) sulphuric acid, was used (4), sensitivity was adjusted to produce 50% of full-scale deflection, and complete spectra were recorded at 2-minute intervals for 30 minutes. With 436 mμ excitation, maximum variation from the mean peak intensity was 1.14% (C.V.).* With 455 mμ excitation (to study the stability of the xenon continuum), maximum deviation was 1.57% (C.V.). Further confirmation of instrumental stability was provided by the congruence of the 15 curves obtained in each of the two test periods. Instrument III was tested with a solution of estrone (0.3 μg/ml) in 80% (v/v) sulphuric acid (4) with 435 mμ excitation and 480 mμ fluorescence. Random fluctuation of the instrument about the peak value amounted to 0.5% (C.V.).

*Coefficient of variation.

Sensitivity

Instrumental sensitivity is measured by the signal-to-noise ratio at an acceptable resolution. "Noise" is defined for practical purposes as the range of random pen motion on the recording chart when the photocell is in the dark (5). A clear distinction must be drawn between a signal due to fluorescent contaminants in the sample (which is background fluorescence and not "noise") and signals generated within the instrument by the design and operation of the optical and electronic components.

While instrument I was available, no precise measurements of sensitivity in terms of signal–noise ratio were performed. An estimate can be derived from the fact that no noise was detected when an estrone solution of 0.17 μg/ml treated by the Bates and Cohen procedure (2) was scanned. The measurements were made at a slit width of 0.1 mm and 87% of maximum sensitivity. The spectral peak was 80% of full-scale deflection.

With instrument II, a solution of 0.34 μg/ml of estrone in 80% (v/v) sulphuric acid, exciting radiation at 436 mμ and the analyzing monochromator at 485 mμ with a slit width of 0.33 mm and maximum gain, the excursion of the recorder pen over 30 seconds was measured. The ratio of fluorescent intensity to pen excursion was 19:1.

Instrument III achieved a signal–noise ratio greater than 14:1 at a concentration of 0.004 μg estrone/ml.

Resolution

Resolution is defined for present purposes as the capacity for the separation of mercury lines at 404.6 mμ and 407.8 mμ. With instruments I and II a metal reflector was placed in the sample cuvette and a relatively broad band in the 405-mμ region of the mercury spectrum projected from the excitation monochromator into the analyzer. With instrument I, resolution of these lines was achieved with a 0.05-mm slit; with instrument II, resolution of the two lines was obtained with 0.02-mm slit. Instrument III was unable to resolve the peaks of the estradiol-17β, 85% (v/v) sulphuric acid spectrum (448 and 475 mμ). The 404.6 and 407.8 mercury lines could not be separated. This is to be expected since a slit of 0.2 mm is required for a separation of 2 mμ, given the grating dispersion of 10 mμ/mm and the smallest slit available in the model tested was 0.4 mm.

Extreme Bandwidth

Extreme bandwidth is defined as the extreme wavelength range of a detectable signal under the given experimental conditions and is used as an operational criterion instead of the customary half-band width. In spectrophotometry the half-band width serves adequately to describe the distribution of radiation. With spectrofluorometers, however, there arises the added necessity of *total* separation of the exciting and fluorescent radiations. Since the exciting light is of far greater intensity, even a small overlap of the "toe" of the excitation spectrum with the fluorescence spectrum will produce serious complications. The purity of radiation emitted by the excitation monochromator was studied by placing a metal reflector in the sample holder, and examining the reflected

radiation at the maximum resolution of the analyzing monochromator. The effect of slit width in the excitation monochromator at various wavelengths is shown, for instruments I and II in Table I. Instrument III has fixed slits 0.4 mm and this type of measurement could not be made.

TABLE I

Spectral characteristics of exciting radiation versus slit width of instruments I and II

Excitation wavelength, mμ	Exciting monochromator slit width, mm	Extreme bandwidth, mμ	
		Instrument I	Instrument II
250	0.25	7	—
	0.50	11	1
	1.00	19	3
	1.50	24	4
300	0.25	45	2
	0.50	35	3
	1.00	39	4
	1.50	47	6
350	0.25	45	2
	0.50	39	2
	1.00	58	4
	1.50	92	5
400	0.25	33	3
	0.50	71	3
	1.00	91	4
	1.50	109	7
450	0.25	43	5
	0.50	67	5
	1.00	95	6
	1.50	158	6

Range of Undistorted Gain

Range of undistorted gain is defined as the range of estrogen concentrations brought to the same scale intensity by changing electronic amplification. For instrument I, estrone in 85% (v/v) sulphuric acid was prepared in concentrations from 1.25 to 0.025 μg/ml. By changing electronic amplification, the peaks of all solutions were brought to the same intensity (80% of full scale) and the spectra were found to be superimposable. This time, unfortunately, the maximum concentration at minimum gain that could be superimposed was not determined. Hence it is only possible to state that the range of undistorted gain was at least 20-fold, and probably much more. With instrument II, concentrations of estrone in 80% (v/v) sulphuric acid from 3.4 to 0.13 μg/ml were tested in a similar fashion. The incident light was at 435.8 mμ, slit width on DK-2 at 0.6 mm, 100% Dial at maximum, and the photomultiplier at 20 X. The concentration of 3.4 μg/ml represented the concentration at minimum sensitivity which produced 85% of full-scale deflection. At maximum sensitivity, this deflection was maintained by a concentration of 0.167 μg/ml and lower concentrations showed a drop in signal height. Thus, this instrument shows a 20-fold range of undistorted gain under these experimental conditions. With instrument III, the incident light was 435–436 mμ, the emitted light at

480 mμ, slit program number 3, and the meter multiplier from 0.001 to 1.0. Superimposable curves could be obtained with estrone in 80% (v/v) sulphuric acid over the range 0.01 to 5 μg/ml, a 500-fold range.

Discussion

On the basis of experience gained in the design, construction, and evaluation of these three instruments, certain comparisons of their characteristics are possible. These are shown in Table II.

It must be kept in mind that the nature of a particular problem in fluorometry may determine the desirable instrumental properties. For example, in the study of reaction kinetics using a fluorometric end point, the problem of instrumentation is relatively simple if a single fluorescence wavelength observed continuously or at intervals yields adequate information. If, on the other hand, the entire fluorescence spectrum must be observed or recorded continuously, another instrumental design is required. In some cases maximum sensitivity is necessary, perhaps at the sacrifice of resolution; in others the reverse may be true. Thus we wish to emphasize that no single spectrofluorometer design will meet all requirements at an acceptable cost. In some circumstances only methodological exploration need be carried out with a spectrofluorometer, while subsequent quantitative or routine determinations may be performed more simply and inexpensively on a filter fluorometer of high sensitivity with carefully selected filters instead of prisms or gratings.

The requirements of instrumental resolution deserve some comment. Although fluorescence emission of irradiated solutions at ordinary temperatures does not have the complexity or sharpness of many other forms of radiant energy because of the nature of the radiative process, there are nevertheless weighty reasons for demanding relatively high resolution in a spectrofluorometer. In the first place, separation must be achieved between the range of "extreme bandwidth" of the exciting radiation and the fluorescence spectrum. In the second place, polycyclic organic compounds are known which have several fluorescence maxima only 3 to 4 mμ apart (14), thus making resolution of this order valuable. Lastly, problems may well occur in which the identification of several compounds in a fluorescent mixture may depend upon fluorescence maxima lying close together; here again, resolution might well be the limiting instrumental factor.

In the instrumental evaluation, "extreme bandwidth" rather than the customary half-band width has been employed. The resolution of both the excitation and the analyzing monochromator is of great importance in the performance of a spectrofluorometer. In contrast to the problems of spectro*photo*meter design, however, the half-band width is not an adequate criterion of instrumental characteristics. The extreme intensity of the exciting radiation compared with the fluorescence makes it imperative to provide sufficiently "pure" exciting radiation and sufficiently great dispersion in the analyzing monochromator as well, so that no overlap occurs between the two even when the wavelengths are relatively close together. In simple fluorometers this can

TABLE II

Summary of characteristics of the three instrumental designs

Advantages	Disadvantages
Instrument I	
1. Commercially available components except for the fluorescence sample holder, lamp shield, and blower	1. High cost for an instrument not readily convertible to absorption spectrophotometry
2. High-intensity light source, providing the mercury lines as well as the xenon continuum	2. Requires construction of several components as well as over-all assembly
3. Exciting radiation of high purity	3. High resolution is achieved at the cost of diminished sensitivity
4. Double-pass analyzing monochromator, giving extremely high resolution with use of a quartz prism	4. Use of a prism-type analyzer avoids overlapping spectra of higher orders, but results in nonlinear wavelength scale
5. Great flexibility of resolving power due to variability of gain, amplifier band-pass ("response"), and wavelength drive speed	5. The strip chart recorder is less convenient than the flat-bed type
6. A-c. signal output, thus simplifying amplification	6. The system is based on a 13-cycle chopper and matched linear amplifier. Such low-frequency units are primarily designed for slow-response detectors such as thermo couples used in infrared work. Stable, linear amplification of a photomultiplier signal could be achieved more simply and at less cost using higher frequency systems
7. Up to 80 db amplification	
8. Standard, commercial wavelength drive and recording system, integrated with the analyzer	
Instrument II	
1. Commercially available units except for a simple cell compartment and a lamp housing; requires minimal assembly	1. Requires construction of several components
2. Light source of high intensity, providing both mercury lines and xenon continuum	2. Sensitivity slightly less than instrument I, much less than instrument III
3. Reliable recording spectrophotometer of high resolution, equally useful for absorption spectrophotometry	3. Limited amplification of d-c. signal
4. Calibrated flat-chart recording, with a scale expander	4. Unshielded cell compartment
5. Precise and variable slit control	
Instrument III	
1. Commercially available, ready-to-use instrument at moderate cost	1. Arc width of light source essentially determines resolution, which is further limited by availability only of fixed slits
2. Light source requires no special cooling. Xenon–mercury source available	2. Because of instrumental design (using arc as slit), instability in arc will cause more serious variations than in instruments I and II
3. Simple optical design and low resolution give extremely high sensitivity (almost 85 times that of instrument II)	3. Gratings have low dispersion, poor resolution in comparison to instruments I and II; may be important in some applications
4. High range of gain (500×)	4. Internal scatter of instrument is relatively high
5. Excitation or fluorescence spectrum can be observed by oscilloscopic display, photometrically or with a strip chart recorder	5. Wavelength drum calibrations do not permit high accuracy of setting or reading

be achieved with comparative ease, using such devices as that of Slaunwhite (15) where the exciting radiation is polarized with a polaroid filter and the fluorescent radiation passed through a crossed polarizer. This method can be used only under certain circumstances and with fluorometers of high sensitivity, but when feasible it is an inexpensive and highly efficient means to obviate overlapping of the two radiations. However, the use of polarizing screens is often not practicable, and consequently greater attention must be paid to the resolution of the monochromators. This is best expressed by the extreme bandwidth, as defined above.

The usefulness of a wide range of gain without change in instrumental resolution (as by change of slits) has been clearly shown in studies of self-absorption phenomena of corticosteroids (9).

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ANTIFUNGAL ACTION OF SALICYLANILIDE. II[1]

R. S. Baichwal,[2] R. M. Baxter, S. I. Kandel, and G. C. Walker

Abstract

The fungistatic activities of certain compounds structurally related to salicylanilide suggest the possible involvement of hydrogen bonding of the anilide hydrogen atom and chelation by the phenolic and amidic oxygen atoms with an enzyme(s)/enzyme system of the fungus. The inhibition of enzymes has been followed through the development of specific color reactions on salicylanilide assay plates. The participation of sulphydryl groups in the mechanism of action is suggested by these tests.

Introduction

Recently, derivatives of salicylanilide possessing anti-infective properties have found use in the treatment of superficial mycoses caused by the dermatophytes (1). Although some attempts are being made to study the mechanism of antifungal action of salicylanilide (1) data relating to the structural specificity essential for the biological activity exhibited by this agent seem to be lacking. Furthermore, comparative figures obtained under similar test conditions for the antifungal activity of compounds whose structure may be correlated with activity are unfortunately not available. Since such a study might be useful in explaining, at least partially, the mode of action of salicylanilide, a number of compounds related to it, whose activity might reflect on the structural features essential for the action of salicylanilide, were prepared and tested for their antifungal action.

The results suggested that the mechanism of action of salicylanilide may involve the blocking of certain enzyme(s) or enzyme systems of the fungus through hydrogen bonding. The present communication reports staining experiments with active groups concerned with oxidizing and reducing substances and systems.

The presence of a number of enzymes in species of dermatophytes has been reported by Tate (2), Bentley (3), and Chattaway *et al.* (4). However, many difficulties are generally encountered in biochemical studies with the dermatophytes (5). Hence the role of salicylanilide with respect to enzyme inhibition was examined only in a qualitative manner by techniques which have usually been employed (6, 7) for studies of the mechanisms of action of penicillin on susceptible organisms.

Methods and Materials

Organism

Trichophyton mentagrophytes 183, supplied by the Ontario Department of Health, was used in all tests. A conidial suspension containing approximately five million conidia per milliliter prepared from stock cultures grown on agar medium for 3 weeks was used as inoculum.

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[2]Present address: Department of Chemical Technology, Matunga Road, Bombay 19, India.

Media

(*a*) Media containing 2% glucose and 1% bactopeptone; pH 6.5–6.7 (broth media).

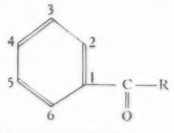
(*b*) Broth media solidified with 2.5% Difco agar.

Test Compounds

Salicylanilide was obtained from L. Light and Co., Ltd., Colnbrook, Bucks. 5-Chloro-, 5,3′-dichloro-, and 5,4′-dichloro-salicylanilides were kindly supplied by Wallace and Tiernan Inc., Belleville, New Jersey. The other compounds tested for their antifungal activities were either obtained from commercial sources or synthesized according to the procedure described in the reference indicated against the respective compound in Table I. *O*-Methyl salicylanilide was prepared by treatment of salicylanilide with diazomethane in the usual manner. The melting point of the resulting compound insoluble in alkali corresponded to that reported (11).

TABLE I

Fungistatic activity of salicylanilide and its related compounds against *T. mentagrophytes**



No.	Substituent	R	Method of preparation reference	Minimum inhibitory concn., μg/ml
1	2 —OH	—NHC_6H_5	*b*	8–10
2	2 —OH;5 —Cl	—NHC_6H_5	*b*	6
3	2 —OH;5 —Cl	—NHC_6H_4-3′Cl	*b*	3–4
4	2 —OH;5 —Cl	—NHC_6H_4-4′Cl	*b*	3–4
5	2 —OH	—NH_2	*b*	110[a]
6	2 —OH	—OH	*b*	138[a]
7	2 —OH	—OC_6H_5	*b*	53
8	2 —OCH_3	—NHC_6H_5	11	45
9	2 —OH	—NC_6H_5 | CH_3	9	130
10	—	—NHC_6H_5	12	118[a]
11	2 —COOH	—NHC_6H_5	13	73
12	4 —OH	—NHC_6H_5	14	96
13	2 —SH	—NHC_6H_5	15	40
14	Aniline		*b*	186[a]
15	Acetoacetanilide		16	106

*Broth medium, stationary growth conditions.

Reagents

Samples of the chemicals were obtained from commercial sources. The reagents were prepared according to references (6) and (7).

Antifungal Activity

For the determination of the fungistatic activities of the test compounds the tube dilution method was followed. Broth tubes (10 ml) containing varying concentrations of the test substance, obtained by the addition of suitable

aliquots of an alcoholic stock solution, were inoculated (0.1 ml) with the test organism and incubated for 3 weeks at $27 \pm 2°$ C. The minimum inhibitory concentrations found are recorded in Table I.

pK_a Value

The pK_a values of salicylanilide and its chloro derivatives were determined potentiometrically by the method reported by Stockton and Johnson (8). This technique is employed for water-insoluble weak electrolytes and depends on the pH of buffers at half-neutralization point.

Influence of Subinhibitory Concentrations

For this purpose, different quantities of the stock solution of salicylanilide were added to flasks containing broth medium (30 ml), inoculated with 0.3 ml of the conidial suspension of the test organism, and incubated at $27 \pm 2°$ C. At the end of each week the mycelial mats were collected, dried, and weighed. The results obtained are presented in Table II.

TABLE II

Effect of various concentrations of salicylanilide on the growth of *T. mentagrophytes**

Concentration of salicylanilide, μg/ml	Incubation time, weeks				
	1	2	3	4	5
	Average weight mycelium in mg				
0	77	213.25	189	152	125
2.13	90	237.70	167.5	157	134
4.26	64	210.5	186.5	148	139
8.52	2	2.0	2.0	2	2
17.04	0	0	0	0	0

* Broth medium, stationary growth conditions.

TABLE III

Effects of specific reagents on different parts of salicylanilide-assay plates seeded with *T. mentagrophytes*

Reagent	Group assumed to be active	Inside of zone of inhibition*	Reaction on assay plates	
			Boundary	Outside of zone of inhibition
1. 2,6-Dichlorophenol indophenol	—SH	Deep blue	Colorless	Light blue
2. Potassium ferricyanide followed by ferric sulphate	—SH	Deep green-blue	Light blue	Light green
3. Cobalt nitrate	—SH	Clear	Ppt.	Cloudy
4. Cadmium acetate	—SH	Clear	Ppt.	Cloudy
5. 2,3,5-Triphenyl-tetrazolium chloride	—SH	Clear	Deep red	Red
6. Methyl green	DGS†	Green	—	Pink
7. Malachite green	DGS	Green	—	Faintly pink
8. Janus green B	DGS	Blue	—	Bluish pink
9. Schiff's	Aldehyde	Clear	—	Pink
10. (*i*) Potassium dichromate and mercury chloride	Dienol (o-polyphenols)	Yellow	—	Light brown
11. (*ii*) Ammonium molybdate in acetic acid	Dienol (o-diphenols)	Clear	—	Cloudy
12. Ferric chloride	Dienol (o-diphenols)	Brown	—	Cloudy

*The control plates with agar medium alone or agar containing salicylanilide generally gave similar reactions but with lesser intensity.
†DGS = Dehydrogenase systems.

Reactions with Specific Reagents

Agar plates containing 20 ml of agar medium were seeded with 1 ml of the conidial suspension of the test organism. At the centers of such plates, standard cylinders were placed, filled with a 2% alcoholic solution of salicylanilide, and then incubated at $27 \pm 2°$ C for 48 to 72 hours. Clear zones of inhibition with a diameter of 20 to 25 mm were shown.

The plates so prepared were flooded with the specific reagents and observed for color or other changes after 20 to 30 minutes. In some cases the reagents were drained after the specified period and the plates were gently washed with water and again observed. The reagents used, the presumed active groups reacting with them, and their effects on different parts of salicylanilide plates are recorded in Table III.

Results and Discussion

From the results recorded in Table I it is seen that with the exception of the chloro derivatives of salicylanilide all the other compounds tested were not as active as salicylanilide.

The removal of the hydroxyl group in salicylanilide appears to reduce the activity of the parent compound considerably (Table I, No. 10). A similar effect, though to a lesser degree, is observed when the hydroxyl group is blocked with a methyl group or substituted with a carboxyl or a sulphydryl group (Table I, Nos. 8, 11, 13). The lesser activity exhibited by *p*-hydroxybenzanilide suggests the importance of the position of the hydroxyl group in the molecule (Table I, No. 12).

The amide group appears to be of still greater importance for activity. The substitution of the anilide hydrogen atom with a methyl group curtails the activity of the parent compound considerably (Table I, No. 9). The decreased activity of phenyl salicylate and salicylic acid indicates the importance of the anilid group (Table I, Nos. 7, 6). That it may be the salicylanilide moeity as a whole which is responsible for activity is suggested from the low activity exhibited by acetoacetanilide (Table I, No. 15).

Although the presence of the COHN grouping suggests a possible mimetic action of a dipeptide for salicylanilide no support for this hypothesis was obtained from previous experiments (1). Chaplin and Hunter (9) found that salicylanilide is slightly less associated than benzanilide. This was attributed to the engagement of the amidic oxygen atom by intramolecular co-ordination with the ortho phenolic hydroxyl group. The properties of chelate copper derivatives of the acetoacetanilides were found (9) to support such a view. Hence a potentiality for chelation with the phenolic and the amidic oxygen atoms is recognized for salicylanilide.

These authors (9) further suggested that the association found in salicylanilide is due to formation of a hydrogen bond between the anilide H atom of one molecule and an electron donor atom of the other. Some of the possible ways in which such bridge formation may occur are: (*a*) to the amide N atom, viz. H—H—N, (*b*) to the phenol O atom, viz. N—H—O (phenolic), and (*c*) to the amide O atom, viz. N—H—O (amidic). Method (*a*) is unlikely in

view of the relative weakness of this bond. This is evident from the hydrogen bond energy values and interatomic distance values given by Pauling (10). Methods (*b*) and (*c*) therefore are possible with molecular association by the intermolecular sharing of the anilide hydrogen atom of one molecule with the phenolic or amidic oxygen atom of another.

It is conceivable from the above discussion that in the antifungal action of salicylanilide the hydrogen bond formation by the anilide H atom of the molecule might well take place with an electron donor atom of a polypeptide or an enzyme of the organism. Bridge formation in the manner N—H—N has already been suggested to be unlikely and it would be more so under physiological conditions. On the other hand, the bonding might take place with the amidic oxygen of a peptide—of a vital enzyme system—resulting in the inhibition of the metabolic activities of the fungus; the groups concerned in reversible hydrogen transfer reactions might as well be involved.

Besides this type of linkage, the co-ordinating abilities of the phenolic and the amidic oxygen atoms of the salicylanilide molecule might bring about chelation with a metallic ion. Although no evidence for the formation of a chelate or for the reversal of inhibitory effect with excess of metals was obtainable in the earlier experiments reported (1), it is not unlikely that once a hydrogen bonding between the inhibiting agent and the protein molecule is established in the biological system, conditions may then be favorable for chelation from steric and other considerations.

The enhanced activity of the salicylanilide molecule on substitution of halogen atoms in suitable positions (1) might be explained on the basis of the mesomeric effect of such groups on the hydrogen bonding capacity and/or chelating ability of the new molecules. For example, substitution of a chlorine atom in the meta or para position of the salicyl ring enhances the acidity of the phenolic hydroxyl group which may facilitate chelation. On the other hand, substitution with the electronegative chlorine atoms in the aniline ring, ortho and para positions respectively, increases electronegativity of the N atom of the amide bond which may facilitate hydrogen bonding. The pK_a values of the chloro derivatives of salicylanilide were therefore determined and these were as follows: salicylanilide 7.45, 5-chloro- 6.50, 5,3′-dichloro- 6.45, and 5,4′-dichloro-salicylanilide 6.35. Thus the increasing fungistatic activities (Table I) of the chloro derivatives with decreasing pK_a values is therefore readily understandable. That the two types of linkages discussed above may be extremely important in the action of salicylanilide and the enhanced activity of the chlorosalicylanilides is borne out by careful comparison of the minimum inhibitory concentrations recorded in Table I.

Tests concerning color reactions on salicylanilide assay plates are based on an effect on oxidizing and reducing systems. In normal aerobic respiration of the test organism, as of other organisms, a balance exists between the rate of dehydrogenation of —SH groups effective in hydrogen transfer and the rate of regeneration of such groups (7). Hence there remains in the aerobic cells a reservoir of hydrogen which is made available to rehydrogenate diketones to dienols which are generally protected from irreversible dehydrogenation due to

the presence of —SH groups. If all the sulphydryl groups are converted to S—S, the dienols tend to form more and more irreversibly oxidized quinoids which may catalyze further dehydrogenations.

The reagents considered to be specific for —SH groups in living cells and tissues failed to indicate the presence of such groups in the zones of inhibition. Their presence, however, was revealed on the areas outside such zones where normal growth of the organism appeared (Table III). At least with four reagents, Nos. 1, 3, 4, and 5, the boundaries between the areas of inhibition and normal growth were somewhat intensified. This might suggest an enhanced metabolic activity and growth of the organism in this region which may have been induced by the substatic concentration of salicylanilide present, as a result of a diffusion gradient. This was observed only with difficulty by the visual examination of the plates prior to treatment with the reagents. Tests were therefore carried out to find the influence of subinhibitory concentrations of salicylanilide under stationary conditions of growth. From the mycelial weights recorded in Table II for the samples containing different concentrations of salicylanilide a stimulation of growth in the presence of subinhibitory concentrations was observed.

A decreased dehydrogenase activity was shown (Table III) in the areas inside the zone of inhibition as compared with the areas outside with reagent Nos. 6, 7, and 8. The impairment of the dehydrogenase activity may be the result of the oxidation of the thiol groups in the inhibition zone or due to a diversified set of reactions.

The test with the ferricyanide reagent for the sulphydryl groups or those for the presence of dienols by chromaffin reaction (reagents Nos. 10 and 11) could not be considered very useful when compared with color reactions reported (7) with penicillin assay plates using *Staphylococcus aureus*. This difference may be concerned with the nature of the test organism, a dermatophytic fungus used in these studies.

These latter reactions, in general, suggest that the mode of action of salicylanilide may be related to enzymes containing sulphydryl groups. Although such simple staining experiments with only a few reagents do not in themselves afford strong evidence in this respect. The results are suggestive in light of the discussion respecting the hydrogen bonding and chelating properties of the salicylanilides. The association of the anilide hydrogen atom of salicylanilide with a sulphydryl or phenolic containing compound due to the formation of a hydrogen bond between the anilide H atom of salicylanide and the electron donor atom of an enzyme (—SH or —OH) has already been mentioned. In light of the relatively marked effect on the antifungal activity of salicylanilide by the replacement of the anilide hydrogen with a methyl group (Table I, No. 9) an association of this type would appear to be a distinct possibility.

Summary

The fungistatic activity determined against *T. mentagrophytes* of a number of compounds structurally related to salicylanilide suggests that the OH and the CONH groupings are important for activity.

The possible role of hydrogen bonding between the anilide hydrogen atom of the molecules with an electron donor atom of a polypeptide or an enzyme vitally concerned with the normal metabolism of the fungus is discussed. The enhanced activity shown by the chloro derivatives of salicylanilides and the pK_a values determined for them seem to support such a view.

Certain specific reagents previously employed for cytological reactions in animal and bacterial cells have been used to indicate if inhibition of certain types of enzymes occurred in the presence of salicylanilide. The color reactions observed on the assay plates are discussed in terms of the relative concentrations of the sulphydryl–disulphide groups.

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A COLORIMETRIC METHOD FOR MEASURING INDICAN[1]

A. M. MARKO AND F. B. REYNOLDS

Abstract

A qualitative test for indican (indoxyl-*O*-sulphate, usually as the potassium salt) has been adapted for the quantitative measurement of indican. The method involves the condensation of indican and *p*-dimethylaminobenzaldehyde in the presence of acid to yield an orange-colored compound. When an excess of sodium acetate is added, the orange-colored reaction product is converted to a cherry-red derivative. The effects of time, acids, temperature, and concentration of reagents on the reaction have been studied and optimum conditions have been selected to obtain a standard curve.

Unsatisfactory analytical results are obtained when this method is applied directly to urine samples and a method for the quantitative elution of indican from charcoal columns has, therefore, been devised. With this modification quantitative recoveries have been achieved with indican alone and with indican added to urine samples.

In addition a review of the literature pertaining to indican measurement and indigoid pigments is presented.

Introduction

During glutamic acid analyses of urines from patients with the malabsorption syndrome (1), the development of an intense purple color was noticed when concentrated hydrochloric acid was added to the urines. The malabsorption syndrome includes nontropical sprue of adults and coeliac disease of children. Rimington, Holiday, and Jope (2) isolated indigotin, which is blue, and indirubin, which is red, from the urine of a patient with sprue, after autoclaving the urine in the presence of acid. Varying proportions of these two isomeric pigments impart to a solution varying shades of blue-red or purple color. It is generally accepted that these pigments arise from the oxidation and dimerization of indican (indoxyl-*O*-sulphate).

The early literature is replete with references to indican, usually its measurement with some attempt to determine the source of urinary indican. Many of these reports are conflicting and much controversy has occurred in the past as to whether or not indican is a normal constituent of urine (3). The confusion may be due to two reasons. First, with the exclusion of Sharlit's method (4, 5) which had restricted application, no reliable method for measuring indican had been available, and second, the indigoid pigments produced from indican have been confused with urorosein formed from indoleacetic acid.

As early as 1891 Rosin in a remarkably thorough study (6) noted that under the influence of acid and an oxidizing agent indican was converted mainly to indigotin and some indirubin; more indirubin was formed if the temperature was raised.

In 1882 Nencki and Sieber described the formation of a red color when sodium nitrite and concentrated hydrochloric acid were added to certain

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pathological urines (7). This is commonly called the urorosein reaction. The reaction was studied by Herter (8), who isolated and identified the chromogen as indoleacetic acid. Later it was shown that the same color was obtained from indoleaceturic acid, the glycine conjugate of indoleacetic acid (9, 10). The pigment has been isolated (11) and shown by synthesis to be diindoylmethene (12).

In 1937 Beckh, Ellinger, and Spies (13) introduced a test which was similar to the Nencki and Sieber test and they believed this to be a measure of porphyrin excretion. Watson (14, 15) showed that porphyrins were not being measured and later (16) demonstrated that the red color obtained in the Beckh, Ellinger, and Spies' test was closely related to indirubin.

It should be pointed out that the formation of skatoxyl from skatole is improbable as may be seen from examination of its chemical structure (17). Furthermore, the so-called skatole red is probably a mixture of indigotin and indirubin (10).

Several methods have been proposed in the past for the estimation of urinary indican. Most of these tests are crude and do not satisfy modern quantitative demands. The most frequently used tests (18–25) employ an oxidizing agent such as calcium hypochlorite proposed by Jaffe in 1870 (26) or more commonly ferric chloride as suggested by Obermayer in 1890 (27). In the presence of these reagents and strong acids, indican is oxidized to indigoid pigments which may be measured after extraction into chloroform. Meiklejohn and Cohen (28) have considered the objections to this method in some detail. The same objections apply to modifications of this test in which a milder oxidizing agent such as copper sulphate has replaced ferric chloride (29). Another method introduced by Wang utilizes potassium permanganate (29, 30). Imabuchi (20) and Stanford (31) list the number of different oxidizing agents that have been employed in the past; these include hydrogen peroxide, potassium chlorate, ammonium persulphate, sulphuric acid, osmic acid, nitric acid, chlorine water, bromine water, and potassium perchlorate.

Another variation is the coupling of indican to other compounds in the presence of acids and oxidizing agents. Pigments of red-blue shade may be formed by condensing indican with isatin (31, 32, 33), resorcinol (34), hexylresorcinol (35), ninhydrin (36), thymol (37, 38), and α-naphthol (39). Fearon and Thompson (40) have indicated that such pigments may be obtained by the condensation of indoxyl with various phenols unsubstituted in the para position. These pigments are classified as urocarmines.

From the results obtained by the methods described above, it has become common practice to accept the normal human excretion of indican to be 10 to 20 mg per day (41). Sharlit modified Jolles' method (38) to measure indican in urine (4) and in blood (5). Sharlit's modification revealed that the indican excretion in 24 hours was many times in excess of previously reported results. The range of excretion by normals was found to be 36.8 to 160.0 mg of indican per day. The results obtained by Sharlit have been ignored except where it was adapted for clinical purposes in two instances (28, 42). Recently Feldheim (43) has improved Jolles' method in a different way by carrying out the reaction at 70° C and obtained a range of 29.6 to 80.7 mg indican per day in normal human

beings. Although the daily variation in a single individual was less, the values as a whole were lower than those obtained by Sharlit.

Although Sharlit's method may have been adequate, we did not choose to employ it for the following reasons: first, there is no certainty that the method is specific for indican; second, the calibration curve s not absolutely linear as required by Beer's law (28); third, other substances such as glucose (5) and urinary pigments (28) can give rise to color which must be removed by preliminary treatment with basic lead acetate. Contrary to the claim (28) that indole and indoleacetic acid do not contribute to the color, Franzen and Bremer (44) found that these substances yielded a pale color. We have confirmed this observation and were not surprised since the conditions are suitable for the formation of urorosein from indoleacetic acid.

An additional reason for carrying out this investigation was the report by Dalgleish (3) that indican appeared as an orange-brown spot on paper chromatograms sprayed with *p*-dimethylaminobenzaldehyde (*p*-DMAB) in hydrochloric acid. The paper after drying was then sprayed with a saturated sodium acetate solution and the orange-brown spot turned an intense cherry-red. This apparently unique color change, associated with the finding that most of the colors formed by the reaction of *p*-DMAB with other urinary substances faded after acetate treatment, suggested the possibility of a highly specific method to measure indican. Therefore, conditions were investigated to produce a quantitative method to measure indican by reaction with *p*-DMAB. Unlike other previous methods, the procedure described here offers one advantage in that the reaction is carried out at room temperature. As will be presented later strong acids and heating are detrimental to indican stability, and side reactions with the formation of indigotin and indirubin usually account for the poor recoveries of indican by other methods. Although acid is used in this procedure, the immediate participation of the highly reactive *p*-DMAB in great excess prevents any observable side reactions.

In 1881 Baeyer (45) showed that indoxyl reacts with *p*-nitrobenzaldehyde to yield indogenides. In 1909 Perkin and Thomas (46) reacted a number of aldehydes, *p*-hydroxybenzaldehyde, *p*-nitrobenzaldehyde, and protocatechuic aldehyde, with indoxylglucuronide and obtained indogenine derivatives which exhibited different colors in acid or base. The first mention of the reaction of indican and *p*-DMAB was a qualitative test (47) which was catalyzed by potassium persulphate. Although Gossner (48) studied the reaction between indican and *p*-DMAB, he made no attempt to make it quantitative. He treated urine with *p*-DMAB and then isolated the red pigment by chromatography on alumina. Gossner also synthesized the same compound by direct reaction of indican and then as further proof of the structure he degraded the benzylidene derivative to isatin by oxidation; the isatin was isolated and characterized.

Indican reacts with *p*-DMAB in the presence of hydrogen ions, and inorganic sulphate is split off in the process of condensation. We have characterized the product of the reaction, 2-*p*-dimethylaminobenzylideneindoxyl, by elementary analysis and melting point (49). We have also provided evidence for the electronic structure of the reaction product by ultraviolet and infrared absorp-

tion studies of the product and other derivatives of indoxyl. The indicator quality of this compound is highly interesting and formed the basis of a successful method for the quantitative estimation of indican.

Materials and Methods

Indican was synthesized by oxidation of indole with alkaline potassium persulphate (50). The yield of crude indican was 20.2% and the material was purified by three successive recrystallizations from aqueous ethanol. Although the over-all yield is poor this is a much more convenient way of preparing indican than the isolation from urines of dogs fed indole (19). Paper chromatography of a concentrated aqueous solution of the synthetic indican showed a single spot with an R_f of 0.43 which had light-blue fluorescence using an ultraviolet lamp emitting the short wave length (2537 angstrom units). After spraying with 2% *p*-DMAB in 6 *N* HCl an orange-brown spot appeared which now had an orange fluorescence. Upon drying and spraying with saturated sodium acetate solution the color changed to a cherry-red which fluoresced orange-red.

p-DMAB

Commercial material was recrystallized twice from aqueous ethanol.

Colorimetry

Colorimetric readings were performed using 18×150 mm calibrated test tubes (51) in a Bausch and Lomb Colorimeter "Spectronic 20".

Absorption Curves

Absorption curves were obtained using a Beckman Spectrophotometer, Model DU.

Preparation of Standard Curve and Analyses of Unknowns

To 1.0 ml of aqueous indican solution (0 to 50 μg per ml) was added 1.0 ml of 3% *p*-DMAB in 6 *N* hydrochloric acid and the orange-brown color was allowed to develop for 30 minutes at room temperature. After this time 10.0 ml of 2 *M* sodium acetate in 50% ethanol was added producing a cherry-red color. The contents of the tube were thoroughly mixed and the color intensities were read using a Spectronic 20. The calibration curve obtained from the readings at 520 mμ was used throughout in analytical determinations.

Essentially the same manipulations were used to study the effects of varying conditions and the optical densities were all recorded at 520 mμ.

Analysis of Urinary Indican

Preparation of charcoal.—Charcoal was deactivated with palmitic acid as described by Dalgleish (3). After drying, the deactivated charcoal was suspended in 0.5 *N* sodium hydroxide and allowed to stand for several hours with occasional mixing. The suspension was filtered by suction, washed with additional sodium hydroxide solution, and then washed 10 times with five volumes of water. The alkali-washed charcoal was placed in a bottle, resuspended in water, and stored in this manner.

Preparation of charcoal columns.—Pyrex glass tubing, 9-mm external diameter and 6.5-mm internal diameter, was drawn out at one end to give a constricted elongation 20 mm long and 2 mm in diameter. The over-all length of the glass column was 150 mm. The junction of the tube and constricted end was packed with pyrex brand wool to a height of 5 mm. Then a slurry of charcoal was pipetted into the column, washed down with water, and allowed to settle to a height of 20 mm under a pressure of 50 to 100 mm of mercury. To prevent disturbance of the charcoal bed, the top of the bed was layered with an additional 5 mm of pyrex brand wool.

Elution of indican.—A 1.0- or 2.0-ml sample of indican solution in water or urine was passed through the column of charcoal. The charcoal bed was then washed with two 1-ml portions of water. The effluent from the sample and the washings were discarded. Sugars, salts, urea, aliphatic amino acids, and interfering substances passed through, while aromatic compounds, including indican and urinary pigments, were retained on the charcoal. These aromatic compounds were eluted with three 2-ml portions of 0.7 *M* phenol in water and the eluate collected in a 10.0-ml volumetric flask. When the second portion of phenol solution passed through, the urinary pigments usually emerged from the column and imparted a yellow color to the eluate. The contents of the flask were diluted to the mark with water and a 1.0-ml aliquot was reacted with *p*-DMAB in the manner described for the preparation of the standard curve and analyses of unknowns. Phenol does not interfere in this reaction. From 6 to 12 columns may be handled by one operator. The whole procedure usually takes about 2 hours to complete, but if the glass wool and charcoal have been packed too tightly the time of manipulations may be inordinately lengthened.

Results

Effect of Time on Heating

The effect of heating *p*-DMAB and indican in a boiling-water bath is shown in Fig. 1. When hydrochloric acid is used to catalyze the condensation, the

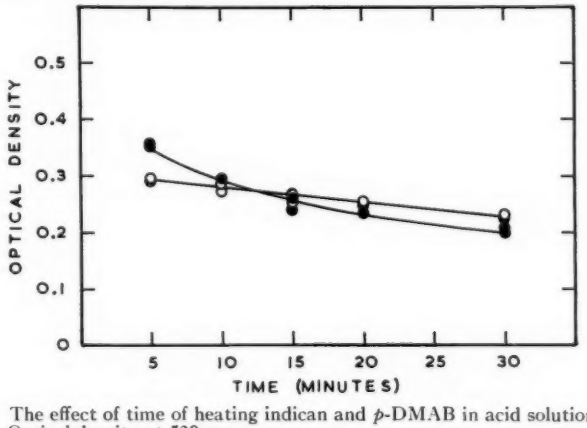

FIG. 1. The effect of time of heating indican and *p*-DMAB in acid solutions: ● HCl, ○ H_2SO_4. Optical density at 520 mμ.

color yield decreases progressively with time. A similar finding results with sulphuric acid but the relationship is linear.

Effect of Time at Room Temperature

It was found unnecessary to use heat to bring about maximum color development and Fig. 2 shows the effect of time on color development at room temperature. A maximum color yield is attained in 30 minutes and remains constant up to 120 minutes. For this reason 30 minutes was used routinely in subsequent assays.

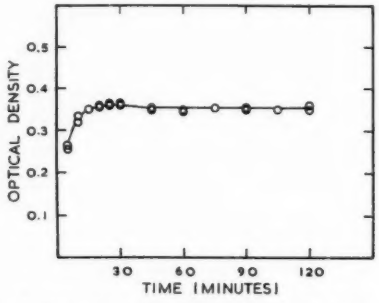

FIG. 2. The effect of time on color development at room temperature. Optical density at 520 mμ.

Effect of Varying Concentrations of p-DMAB

To determine the optimum concentration of *p*-DMAB reagent, varying concentrations of this reagent were prepared and a constant volume added to the indican solution. The results of this experiment are represented in Fig. 3. Up to a concentration of 1.5%, maximum color is not achieved. After that point absorption is linear to a concentration of 4.0%.

Effect of Varying Hydrochloric Acid Concentrations

The effect of varying hydrochloric acid concentrations is illustrated in Fig. 4. There appears to be a region from 6 to 8 *M* acid where the greatest amount of color develops. Below 6 *M* the color production is poor.

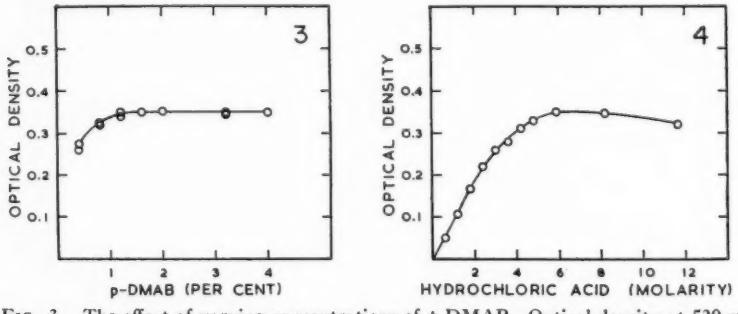

FIG. 3. The effect of varying concentrations of *p*-DMAB. Optical density at 520 mμ.
FIG. 4. The effect of varying hydrochloric acid concentration. Optical density at 520 mμ.

Calibration Curve

Optimum concentrations and conditions studied in previous experiments being used, a calibration curve was made and the optical densities recorded at three different wave lengths. It can be seen from examining Fig. 5, that there is linear relationship between optical densities and indican concentrations varying from 0 to 50 μg per ml. The greatest intensity is obtained at 520 mμ, which is the absorption peak of the red pigment. With sulphuric acid as the catalyst a much lower response is obtained; this effect of sulphuric acid is unexplained. It was found that indican in aqueous solutions underwent a slow destruction and it is important to use freshly prepared indican standard solutions.

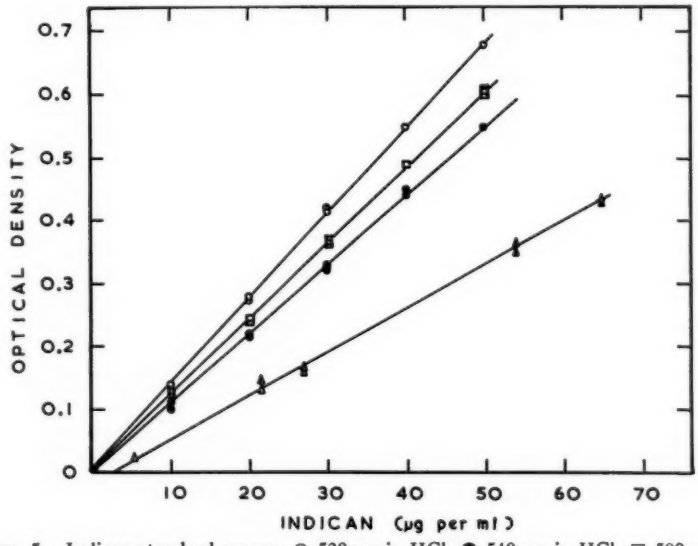

FIG. 5. Indican standard curves: ○ 520 mμ in HCl, ● 540 mμ in HCl, □ 500 mμ in HCl, △ 520 mμ in H_2SO_4.

Absorption Curve of Indican

The ultraviolet absorption curves of indican in water, in 0.1 *M* acetic acid, and in saturated borate buffer at pH 10.8 were carried out. The shapes of the curves were the same with a maximum peak at 279 mμ and a minimum at 240 mμ. In the presence of acetic acid the minimum was shifted to 246 mμ. The major peak had two shoulders, one with a plateau from 273 to 275 mμ and a sharp spike at 288 mμ. In water and acetic acid the molar extinction coefficient was 5460 while in borate buffer it was increased to 5720.

Urinary Analysis of Indican

When the method was applied directly to urine samples, one of three possible results was obtained: first, no color development of any kind; second, development of a muddy brownish-pink color; or third, development of a typical color but of lower intensity. Some urines contained an interfering substance which

to date has not been identified. For this reason, attempts were made to combine chromatography with the method. Indican could be eluted from Dowex-50 in the hydrogen ion form but with variable recoveries from 40 to 100%. The reason for the loss of indican is that at low pH indican is probably converted to indigoid pigments. Using Dowex-50 resin in the potassium form improved the recoveries from 81 to 100%. Polyamine and quaternary resins bound indican irreversibly and could not be used.

The use of acid alumina columns gave recoveries of 39 to 75%. Again the loss was probably due to acid destruction of indican.

Next recoveries from columns of charcoal deactivated with 4% palmitic acid were studied and gave recoveries from 71 to 92.5%, with an average of 88.4%. The loss here again was attributed to the low pH of the deactivated charcoal.

Completely quantitative results were obtained if the deactivated charcoal was washed with dilute sodium hydroxide followed by water to remove the excess alkali. Table I summarizes the recoveries of indican from alkali-washed charcoal columns.

TABLE I

Recoveries of indican

Amount of indican, μg	% recovery
23.0	101.5
23.0	99.5
23.0	94.5
23.0	97.8
23.0	103.8
23.0	95.4
57.5	101.0
57.5	97.2
57.5	100.0
57.5	100.5
57.5	95.3
57.5	103.2
57.5	101.5
	Average 99.3
Recovery of indican added to urine	
27.0 added to urine (in triplicate)	96.7
27.6 added to urine (in duplicate)	100.8
(in duplicate)	99.8
	Average 99.1

For two different amounts of indican, 23.0 μg and 57.5 μg, the recovery of indican varied from 94.4 to 103.8% with an average of 99.3%. The recovery of indican added to different urines gave recoveries from 96.7 to 100.8% with an average of 99.1%. The over-all recovery was 99.2%.

In Table II are given results on the daily urinary indican excretion by normal individuals. Unlike the commonly accepted figures of 10 to 20 mg per day (41),

the range with this method is 32.0 to 142.2 mg per day and confirms the high values observed by Sharlit (4).

TABLE II

Indican content of normal human urine

Subject	Date	Indican per day, mg	Indican per ml, mg / Creatinine per ml, mg
G.H.	Aug. 28–29	110.6	0.051
G.H.	Aug. 29–30	63.0	0.034
F.R.	July 9–10	83.6	0.035
F.R.	July 10–11	142.2	0.067
L.J.	Aug. 20–21	54.1	0.036
L.J.	Jan. 7–8	63.2	0.028
J.G.	July 8–9	55.5	0.033
H.G.	Nov. 5–6	83.5	0.078
H.G.	Nov. 7–8	32.0	0.023
A.M.	Nov. 5–6	78.4	0.038
A.S.	Nov. 6–7	44.5	0.041
A.S.	Nov. 7–8	47.3	0.027
Average			0.043

When results are expressed in relation to creatinine content of urine, one obtains indican: creatinine ratios ranging from 0.023 to 0.078 with a mean of 0.043. There is considerable variation not only among different individuals but also day-to-day variations in the same person.

The method is specific for indican for the following reasons:

1. The recoveries of indican alone or indican added to urine are quantitative.

2. Paper chromatography of urines with high contents of indican show only a single trailing spot which gives typical color changes (orange-brown to cherry-red) and has an identical R_f value to that of pure indican.

3. When indoles of urine were concentrated 10-fold (3) and subjected to paper chromatography, in addition to the indican spot, five faint blue spots were visible. The intensity of the blue faded after spraying with sodium acetate solution. In the colorimetric method urine was diluted ultimately 60- to 120-fold so the contribution of other indoles to the final color was negligible. Furthermore, at 520 mμ the contribution of any faint blue color was insignificant compared with that of the indican color.

Acknowledgments

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THE AMINO ACID CONTENTS OF HORSE GLOBIN AND OF ITS COMPONENT POLYPEPTIDES[1]

A. F. S. A. HABEEB[2] AND DAVID B. SMITH

Abstract

Horse globin and its component polypeptide chains obtained by fractional precipitation and column chromatography have been analyzed for their constituent amino acids. The principal difference between the two chains is that the valyl-leucyl chain is rich in serine and threonine and poor in glutamic acid and tryptophan compared to the valyl-glutaminyl chain.

Introduction

Horse globin has been separated recently into two polypeptide chains with the N-terminal sequences valyl-leucyl (*vl*-chain) and valyl-glutaminyl (*vg*-chain) (1). As a preliminary to sequence study on these materials an accurate amino acid assay is required. An analysis reported by Tristram (2) sums to a molecular weight of about 58,300, a value significantly lower than the molecular weight of about 64,000 computed from the molecular weight of horse hemoglobin (3).

The present paper describes the amino acid analysis of horse globin and its component polypeptides, by the ion exchange method developed by Moore, Spackman, and Stein (4).

Methods

Horse oxyhemoglobin and globin were prepared from fresh citrated blood as already described (5).

The *vl*- and *vg*-chains were separated by fractional precipitation with 1.5% HCl–acetone following the procedures described by Wilson and Smith (1) except that 1480 ml acid–acetone rather than 1600 ml was added to 400 ml 0.5% globin solution in the initial precipitation. An improved separation of the two chains was obtained thereby. After subfractionation, the final yield of *vl*-globin was 8% and of *vg*-globin, 18%.

These preparations were assayed (1) for the second amino acid residue from the N-terminal ends of their constituent polypeptides in order to confirm their identity and indicate their purity. In the *vl*-preparation, 4.5% of the residues in the second position was glutamic acid, the remainder being leucine. In the *vg*-preparation, glutamic acid with only negligible amounts of leucine were found in this position.

Globin was also fractionated following the chromatographic method described by Wilson and Smith (1). The same three fractions, Nos. 1, 2, and 3 respectively, were obtained as the concentration of urea in the eluting solvent was raised from 2 *M* to 8 *M*.

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[2]National Research Council Postdoctorate Fellow 1958–1959.

Amino acid analyses were carried out on columns of Amberlite IR-120 resin prepared according to Moore, Spackman, and Stein (4). All determinations were in duplicate.

Samples of protein were hydrolyzed in evacuated sealed tubes with glass-distilled 6 *N* HCl at 105° C for 24 and 72 hours. An aliquot of the hydrolyzate was analyzed for nitrogen and the quantity applied to the column was calculated on the basis of 16.8% nitrogen in globin.

Cysteine was determined both as cysteic acid and S-carboxymethyl cysteine. Samples were oxidized by performic acid as described by Schram, Moore, and Bigwood (6) and cysteic acid was determined by chromatography at room temperature on a 15×0.9 cm column of Amberlite IR-120 by using 0.2 *M* citrate buffer, pH 3.25, as eluent. When 1-ml fractions were collected, cysteic acid emerged in the 8th to 12th fractions and was well separated from other amino acids. Recovery of cysteic acid was assumed to be 82% (7). S-carboxymethyl cysteine was obtained by reducing the protein in 0.5% solution with potassium borohydride in the presence of urea (8) followed by treatment with iodoacetic acid. After exhaustive dialysis against water and lyophilization, hydrolysis was done in evacuated sealed tubes for 24 hours and S-carboxymethyl cysteine was determined by chromatography on 150×0.9 cm columns with 0.2 *M* citrate buffer, pH 3.25, as eluent.

For chromatography of the basic amino acids, BRIJ solution (4) was not added to the eluting buffer.

Tryptophan was determined by the spectrophotometric method of Bencze and Schmidt (9) in 0.1 *N* NaOH with a Cary automatic recording spectrophotometer.

Results and Discussion

Table I includes the yields of amino acids obtained from whole globin and *vl*- and *vg*-globins prepared by fractional precipitation. The results for whole globin are expressed as residues per 64,000 g and for the separated chains as

TABLE I

Amino acid analysis of horse globin and its *vl* and *vg* polypeptide chains

Amino acid	Globin (residues/64,000 g)			Valyl-leucyl chain (residues/32,000 g)			Valyl-glutaminyl chain (residues/32,000 g)		
	24 hours	72 hours	Assumed No.	24 hours	72 hours	Assumed No.	24 hours	72 hours	Assumed No.
Aspartic acid	56.5	56.8	56	28.4	29.1	28	27.8	28.0	28
Threonine	22.6	21.0	24*	17.3	16.1	18*	5.8	4.8	6*
Serine	32.3	21.3	38*	22.2	17.5	24*	10.4	7.2	12*
Glutamic acid	36.9	36.3	36	10.0	10.5	10	26.5	26.8	26
Proline	20.3	15.0	22	11.7	12.5	12	9.7	9.8	10
Glycine	51.7	50.5	50	21.7	21.7	22	27.5	27.8	28
Alanine	63.5	63.5	64	35.8	35.0	36	28.1	29.0	28
Valine	58.2	61.6	62	25.6	27.7	28	32.5	36.0	36
Methionine	4.5	5.0	4	2.5	2.2	2	2.0	2.2	2
Leucine	84.3	86.0	84	46.2	46.3	46	37.2	36.7	38
Tyrosine	12.5	11.9	12	6.5	5.5	6	6.1	5.5	6
Phenylalanine	30.4	30.8	30	14.4	14.8	14	15.3	15.3	16
Lysine	43.2	41.8	44	22.0	22.3	22	21.4	21.9	22
Histidine	35.4	38.1	38	20.7	20.5	20	17.4	17.7	18
Arginine	13.8	14.4	14	6.0	5.8	6	7.6	7.8	8
Cysteic acid	3.9	—	4	2.0	—	2	2.2	—	2
S-Carboxymethyl cysteine	2.8	—	—	1.6	—	—	1.1	—	—
Tryptophan	—	—	8	—	—	2	—	—	6

*Value extrapolated to zero time of hydrolysis.

residues per 32,000 g. Also included are the number of residues obtained on adjusting the analytical results to the nearest even number. The values for serine and threonine were obtained by extrapolation to zero hydrolysis time. This method of expression emphasizes the close accountability of the amino acid content of whole globin in terms of equal amounts of the two chains. A choice of an even number of residues is indicated by the analytical results and conforms with molecular weights for both chains of about 16,000 (5).

Valine was found to increase on hydrolysis for 72 hours. This may indicate the presence of valyl-valyl linkages known to be resistant to acid hydrolysis. The variations in leucine, histidine, and glycine are within experimental error. The error in proline estimation was higher than that with the other amino acids owing to its very low color yield (leucine equivalent 0.225). Some of the methionine values were high because of errors in the base line with change in the eluting buffer from pH 3.25 to pH 4.25.

Tryptophan in *vl*-globin amounted to 1.76 to 2.7 residues. The higher value was obtained from the ultraviolet absorption curve while the lower value was obtained from the absorption curve after adding tryptophan to bring the tyrosine–tryptophan ratio to 1:1 (9); this lower value is considered the more reliable.

Values of carboxymethyl cysteine were much lower than those obtained with cysteic acid and the former method does not seem useful for determination of cysteine in horse globin. The cysteic acid method indicated four residues. By titration with methyl mercury, Hughes (10) found that horse globin contained four sulphydryl groups. Ingram (11) found that native horse globin had four sulphydryl groups available to silver nitrate but that hemoglobin denatured with sodium dodecyl sulphate had six groups. This high value may be due to some other group titrating with an affinity for silver close to that of sulphydryl (12).

From Table I and Fig. 1 (showing the amino acid pattern obtained with the *vl*- and *vg*-chains) it appears that the most significant differences between the two chains are in the serine, threonine, glutamic acid, and tryptophan contents. The higher values of serine and threonine and the lower value of glutamic acid in *vl*-globin contrast with the low ratio of serine and threonine to glutamic acid found in *vg*-globin.

Since the tyrosine content of both the *vg*- and *vl*-chains was the same, the higher content of tryptophan in the former was forecast in the lower tyrosine–tryptophan ratio noted earlier (13).

The single residue of cysteine in each chain also confirms an earlier report (13). There is only one residue of methionine in each chain. Noteworthy is the complete absence of isoleucine.

Table II gives the analysis of the three fractions obtained by chromatography of globin. The composition of the material of the first fraction was identical within experimental error with that of *vl*-globin obtained by fractional precipitation (Table I). Fraction 2 similarly has the same content as the *vg*-globin (Table I) with the exception that glutamic acid was slightly lower.

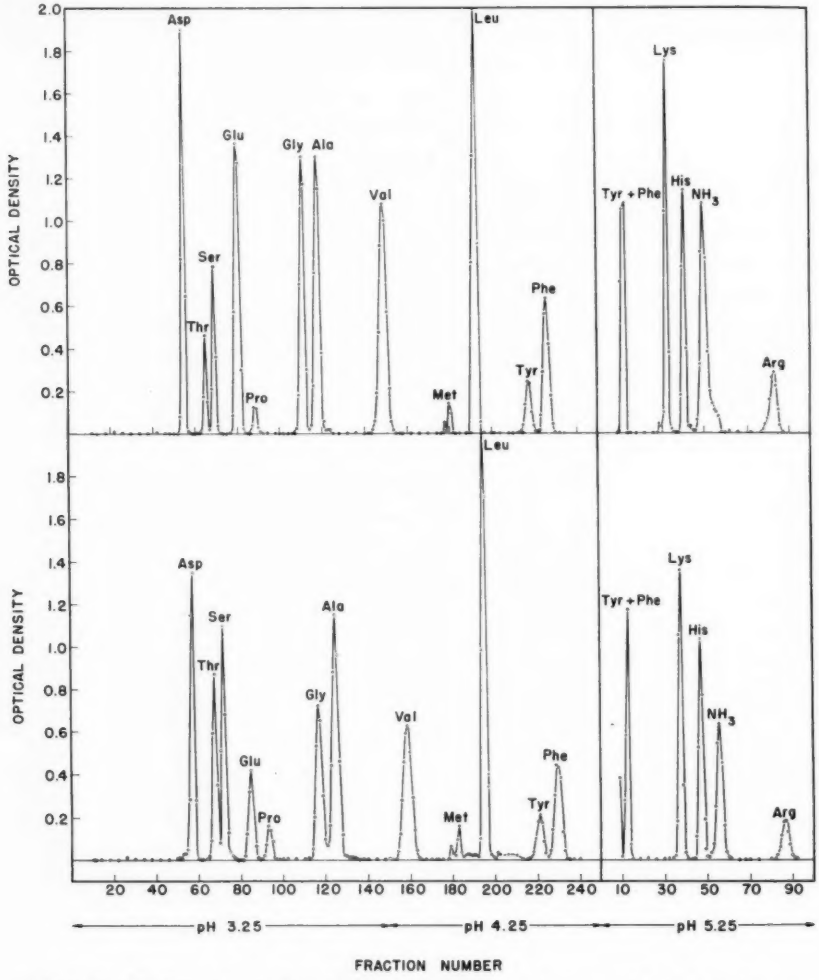

FIG. 1. Elution patterns from ion exchange columns of amino acids in hydrolyzates of horse globin components. Upper, elution patterns of *vg*-globin; lower, elution patterns of *vl*-globin.

The protein of fraction 3 differed slightly from the *vg*-globin prepared by fractional precipitation in the higher serine and threonine and lower glutamic acid contents of the former. These discrepancies could arise from contamination with undissociated globin, the *vl*-component of which would affect these three amino acids chiefly and in the direction found. Wilson and Smith (1) found that the third fraction contained small amounts of leucine in the second amino acid position. Moreover the slight amount of brown coloration remaining in globin preparations is eluted from the column in fraction 3. This material

TABLE II

Amino acid analyses of fractions obtained from column chromatography with urea pH 1.9

	Fraction number					
	1 (residues/32,000 g)		2 (residues/32,000 g)		3 (residues/32,000 g)	
Amino acid	Found	Assumed	Found	Assumed	Found	Assumed
Aspartic acid	28.0	28	28.0	28	28.0	28
Threonine	17.2	18	7.0	8	8.7	8
Serine	24.6	24	12.4	12	15.1	16
Glutamic acid	10.7	10	22.0	22	22.7	22
Proline	12.3	12	9.6	10	10.0	10
Glycine	21.8	22	25.6	26	25.6	26
Alanine	34.3	34	28.2	28	26.8	26
Valine	25.0	26	33.4	34	29.2	30
Methionine	1.7	2	2.2	2	1.8	2
Leucine	44.0	44	40.2	40	40.0	40
Tyrosine	5.3	6	5.5	6	5.4	6
Phenylalanine	13.4	14	16.5	16	15.3	16
Lysine	21.2	22	21.9	22	21.8	22
Histidine	20.8	20	17.2	18	18.2	18
Arginine	6.1	6	8.0	8	7.9	8

may be residual heme and Reichmann and Colvin (14) have noted that the dissociation of horse globin is incomplete in the presence of heme.

Hill and Craig have recently separated by countercurrent partition methods and analyzed the two types of polypeptide chains in human globin (15). The distribution of some amino acids between the two chains resembles the corresponding distribution in horse globin. In particular, there is a high hydroxy-amino acid/glutamic acid ratio in the valyl-leucyl or α-chain of human globin and a low ratio in the valyl-histidyl or β-chain, which, with a similar situation in horse globin, suggests that this feature is important in the architecture of hemoglobin. Also the human α-chain, like the horse *vl*-chain, has the lower tryptophan content. The similarities between the two proteins extend to the distribution of tyrosine and phenylalanine also.

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THE SULPHYDRYL CONTENT OF β-LACTOGLOBULIN[1]

A. F. S. A. HABEEB[2]

Abstract

The reaction of sulphydryl groups of β-lactoglobulin with N-ethylmaleimide, *p*-chloromercuribenzoate, and iodoacetic acid has been studied. Using N-ethylmaleimide with native and sodium dodecyl sulphate denatured protein, a value of 2.3 groups of sulphydryl per mole of protein was obtained. A value of 2.15 moles of sulphydryl per mole of protein was obtained from reaction with *p*-chloromercuribenzoate but lower values were obtained after urea denaturation. The reaction of iodoacetic acid was followed by the determination of residual sulphydryl by the *p*-chloromercuribenzoate method.

Introduction

Since the guanidination of β-lactoglobulin (LG) with 1-guanyl-3,5-dimethyl pyrazole nitrate required blocking the sulphydryl groups of LG to prevent aggregation (1), the reaction of the sulphydryl groups of LG with reagents, N-ethylmaleimide (NEMI), *p*-chloromercuribenzoate (*p*CMB), and iodoacetic acid (IAC), has been examined.

Methods and Results

Reaction of β-Lactoglobulin with N-Ethylmaleimide

The reaction of NEMI with LG was examined using a modification of the spectrophotometric technique of Alexander (2). A pH of 7.2 was used since at this pH the reaction with sulphydryl was much faster than the decomposition of the reagent (3). In order to minimize interference by substances that absorb at the recommended wave length of 300 mμ (2), the measurements were made at 310 mμ where the absorption was only 5% less.

The reaction was carried out at room temperature by adding 3 ml of NEMI solution (0.5 mg/ml in 0.1 *M* pH 7.2 phosphate buffer) to 3 ml of 1.5% LG solution in phosphate buffer. To determine the rate of decomposition, 3 ml of NEMI solution was added to 3 ml phosphate buffer. The optical density was read against blank solutions of protein and buffer respectively. Experiments were also made with 0.1 *M* sodium dodecyl sulphate (SDS) or 8 *M* urea in all solutions. The protein was kept in urea solution at room temperature for 24 hours before adding the reagent. The plot in Fig. 1 of log optical density against time for NEMI in buffer, and in native and SDS-denatured protein is a measure of the rate of disappearance of NEMI in the corresponding solutions. The relationships are linear in all cases, indicating a first-order reaction mechanism. The quantity of NEMI that reacted with the protein at different times was calculated from the optical density difference obtained from Fig. 1 and from a similar plot for urea-denatured protein. From these values a plot of mole sulphydryl reacted as a function of time was given (Fig. 2). The reaction

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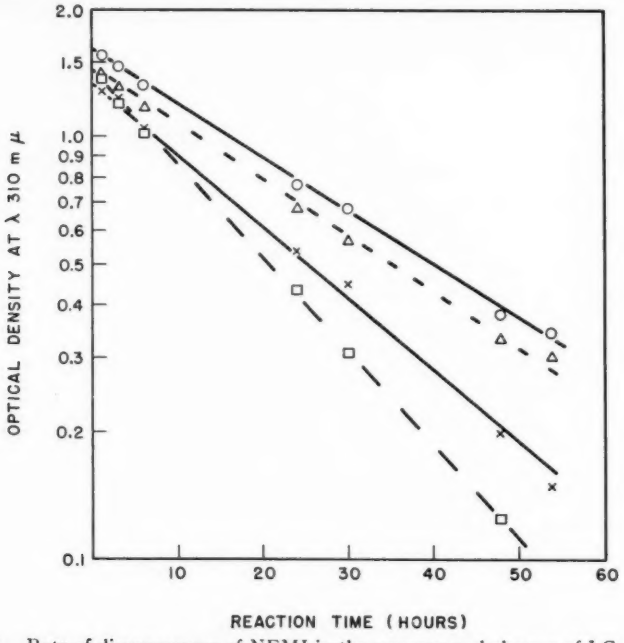

FIG. 1. Rate of disappearance of NEMI in the presence and absence of LG. △—△ NEMI in phosphate buffer; □—□ NEMI in LG solution; ○—○ NEMI in 0.1 *M* SDS; ×—× NEMI in SDS-denatured LG.

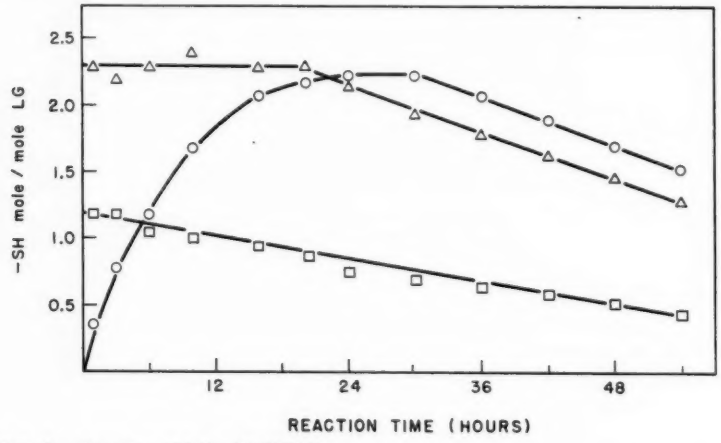

FIG. 2. Reaction of LG with NEMI.
○—○ native LG; △—△ SDS-denatured LG; □—□ urea-denatured LG.

of native LG was very slow but was practically complete after 30 hours with an end value of about 2.3 groups per mole of LG of molecular weight 37,300. LG denatured with SDS gave a similar value after 1-hour reaction and the yield

did not increase with time. This result indicated that denaturation was complete and was accompanied by complete reaction with NEMI. LG denatured with urea for 24 hours gave a value of about 1.2 moles sulphydryl per mole LG. As a result of the instability of NEMI, its concentration in the control and protein solutions is small and considerably different after longer reaction periods. This is reflected in a decrease in the sulphydryl values calculated, as shown in Fig. 2.

The product of the reaction of native LG with NEMI for 30 hours at room temperature moved as a single symmetrical peak upon ultracentrifugation with $s_{20,w}$ 2.46S, at 1% concentration. This suggests that no structural changes occurred in the protein, as the value for native LG is $s_{20,w}$ 2.41S.

Reaction of β-Lactoglobulin with p-Chloromercuribenzoate

The extent of the reaction of LG with *p*CMB was followed by spectrophotometry (4) and by colorimetry with dithizone (5).

Spectrophotometric measurements were made in 0.1 *M* phosphate buffer, pH 6.7, since the turbidity of the solutions at pH 4.7 as recommended (4) affected absorbancy. The procedure was as follows: (*a*) 3 ml *p*CMB solution, containing about 0.1 μmole/ml in 0.1 *M* phosphate buffer, was added to 3 ml buffer and the concentration of *p*CMB calculated from the optical density at 232 mμ using ϵ_M 1.69×10^4; (*b*) 3 ml LG solution containing about 1 mg/ml in phosphate buffer and the concentration of LG determined from the optical density at 280 mμ using $E^{1\%}_{1cm}$ 9.5; (*c*) 3 ml *p*CMB solution was added to 3 ml LG solution and the mixture shaken for various time intervals. The difference of optical density at 255 mμ using appropriate blanks, between (*c*) and (*a*) is a measure of mercaptide formation. The total increase in absorbancy accompanying mercaptide formation was obtained after 24 hours for several protein concentrations in the presence of 0.047 μmole of *p*CMB. The results in Fig. 3

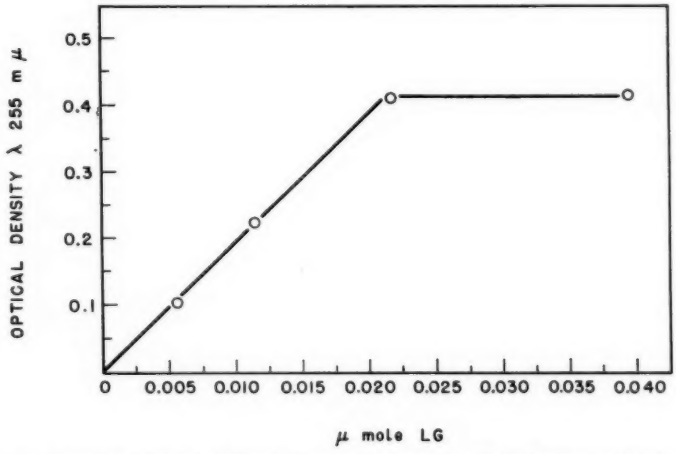

FIG. 3. Reaction of LG at different concentrations with *p*CMB (*p*CMB at 0.047 μmole/ml concentration).

show that at maximum mercaptide formation 0.022 μmole LG reacts with 0.047 μmole *p*CMB, indicating 2.15 moles sulphydryl per mole LG of 37,300.

The reaction of LG with *p*CMB was measured by colorimetry with the dithizone method (5). LG was reacted with *p*CMB in 0.1 *M* phosphate buffer, pH 6.7. At intervals, 1-ml portions of the mixture were withdrawn and shaken with 6 ml of dithizone solution in chloroform together with a drop of N-octyl alcohol to reduce emulsification. The unreacted *p*CMB was calculated from the decrease in optical density at 610 mμ of the dithizone from calibration curves.

The rate of reaction of LG with *p*CMB measured by both methods is shown in Fig. 4. The initial rate obtained by the dithizone method was higher than that given by Boyer's method (4) but the end points were in agreement, giving a value of 2.1 moles sulphydryl per mole LG at about 24 hours.

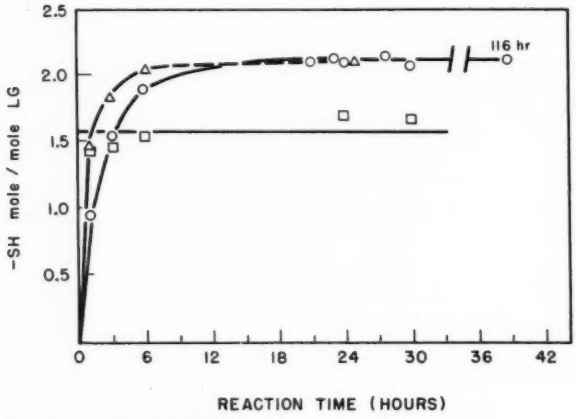

FIG. 4. Reaction of LG with *p*CMB.
○—○ spectrophotometric method; △—△ dithizone method; □—□ urea-denatured LG by spectrophotometric method.

SDS could not be used because it absorbs strongly at 255 mμ and forms a stable emulsion with the organic phase in the dithizone method. The reaction in the presence of 8 *M* urea was studied by adding *p*CMB solution quickly to the freshly denatured protein solution. The dithizone method gave an average value of 0.5 mole sulphydryl per mole LG after 1, 3, 6, and 24 hours. Using Boyer's method (4) a value of about 1.5 moles sulphydryl (Fig. 4) was obtained, which is lower than that for native LG.

Native LG which had reacted completely with *p*CMB at room temperature in 0.1 *M* phosphate buffer, pH 6.7, moved as a single symmetrical peak upon ultracentrifugation with $s_{20,w}$ 2.44S compared with $s_{20,w}$ 2.41S for native LG.

Reaction of β-Lactoglobulin with Iodoacetic Acid

The reaction was carried out at room temperature with 50 and 100 moles IAC per mole LG at pH below 8.5, where groups other than sulphydryl do not react (6, 7). Samples were withdrawn after 1, 3, 6, 10, 24, and 30 hours

and dialyzed exhaustively first against 0.1 *M* sodium chloride then against 0.1 *M* phosphate buffer, pH 6.7. The residual sulphydryl was determined by the modified Boyer's method. The reaction of LG with IAC is slow but complete after 30 hours (Fig. 5).

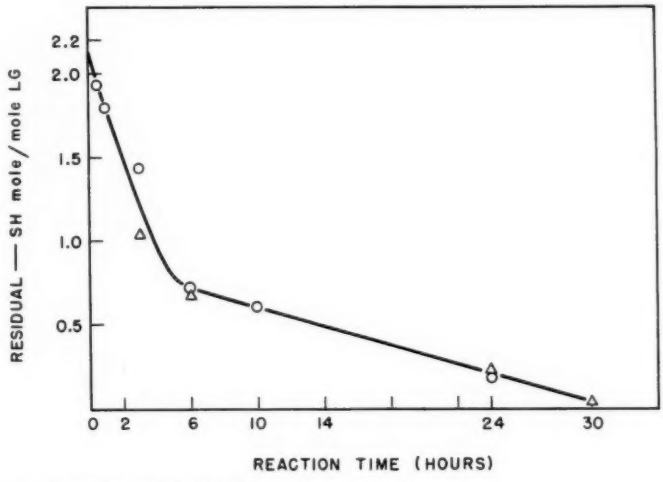

FIG. 5. Reaction of LG with IAC.
○—○ IAC at 50 moles/mole protein; △—△ IAC at 100 moles/mole protein.

Discussion

The reaction of LG with NEMI, *p*CMB, and IAC, although slow, is complete in about 30, 24, and 30 hours respectively. The products resulting from the first two reagents are not structurally different as indicated by the sedimentation pattern.

For the quantitative determination of sulphydryl groups with NEMI, the reaction can be completed in 1 hour following denaturation of LG with SDS and yields the same results (2.3 moles sulphydryl per mole LG) as those obtained with the native protein after 30 hours' reaction. Treatment of LG with SDS did not result in a lower sulphydryl content of the protein. This is in contrast to the work of Allison and Cecil (8), who observed that the thiol groups of hemoglobin were susceptible to oxidation in SDS at pH 9. The fact that NEMI solution was added rapidly to freshly prepared solution of SDS-denatured LG at pH 7.2 may account for this variability. Guanidine hydrochloride was not suitable as a denaturant since it absorbs strongly at 310 mμ.

The reaction of *p*CMB with native LG gives a value of 2.15 moles sulphydryl as determined by both Boyer's method and dithizone. This is in accord with a value of 2.2 moles sulphydryl per mole LG molecular weight 35,000 (4). Fraenkel-Conrat *et al.* (9) obtained a value of 0.5 mole per 10,000 which gives 1.87 moles sulphydryl per mole LG 37,300 by direct titration of LG with *p*CMB using nitroprusside as external indicator. The initial rate obtained by

the dithizone method was higher than that given by Boyer's (4) which was similar to results obtained with milk xanthine oxidase (5) and may be due to denaturation attendant upon shaking with the organic phase.

The use of urea as denaturant to liberate masked sulphydryl groups may not be advisable for all proteins. As is shown in Figs. 2 and 4 with urea-denatured LG the lower values of 1.2 and 1.5 moles were obtained with NEMI and *p*CMB by Boyer's method respectively. These low values are most probably due to oxidation of sulphydryl groups during urea denaturation. Cole *et al.* (10) observed the disappearance of sulphydryl groups of hemoglobin in urea. Similarly Kolthoff *et al.* (11) found that sulphydryl in serum albumin was oxidized in guanidine hydrochloride and that after longer periods of denaturation in the absence of oxygen there is an apparent decrease in sulphydryl attributed to cross linking. The very low value of 0.5 mole sulphydryl per mole LG obtained with urea-denatured LG using dithizone cannot be interpreted from the data obtained.

Figure 5 shows that the reaction of iodoacetic acid with LG is surprisingly slow, requiring about 30 hours for completion. Various workers have observed the variation in reactivity of IAC with sulphydryl of different native proteins and even of simple alkyl mercaptans (12). Whereas complete reaction was obtained with bromelin (13), Rosner (14) found no reaction with native egg albumin at the end of 5 hours at pH 7.3.

Since the sulphydryl groups of proteins show different degrees of affinity for sulphydryl reagents, it is preferable to submit the protein to different reagents. Egg albumin reacts differently with sulphydryl reagents. It reacts with NEMI only when denatured with Duponol (2), while with *p*CMB, reaction is complete in 15 minutes (4) and no reaction takes place with IAC at the end of 5 hours (14). On the other hand it has been found in this work that the sulphydryl groups of LG react uniformly with NEMI, *p*CMB, and IAC.

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SEDIMENTATION OF CRYSTALLINE GLOBULIN FROM CUCURBIT SEEDS[1]

DAVID GENE ANDERSON[2] AND A. G. MCCALLA[3]

Abstract

Crystalline globulins from cucurbit seeds decreased in dispersibility in the order: squash and pumpkin, cucumber, watermelon. Analysis by high-speed sedimentation showed that most of the dispersions in buffers below pH 4.8 contained two components, with sedimentation constants approximately 3 and 7. The proportions of the components varied with species, pH, and time after preparation. The globulin from all species was insoluble in dilute buffers between pH 4.8 and 8.2. A third component, with sedimentation constant approximately 12, was dominant above pH 8.2 but was gradually replaced by the "7" component as the pH was increased. The "3" component was found only at pH 11.9. The globulin from each species is considered to be a single protein with the components forming an association–dissociation system.

Introduction

The properties of crystalline globulins prepared from seeds have been the subject of frequent investigations during the last few years. Earlier work from this laboratory (1) used electrophoretic and sedimentation analyses for studying the crystalline globulin from the seeds of squash (*Cucurbita maxima*). Although several components were present under a variety of experimental conditions it was concluded that the globulin was a single protein that forms an association–dissociation system on dispersion. Kretovich *et al.* (2, and review in 3) also studied the globulins from *C. maxima* (common name given as "pumpkin" in translation). They believe that Osborne's method (4) of preparation, which most investigators have followed, denatures the protein but that a diffusion desalting method of Zelenskii (5) does not. Using Osborne's method, they obtained two globulin fractions, while using Zelenskii's, they obtained four. These investigators used loss of enzymatic activity as a measure of denaturation and, according to Danielson (3), drew unwarranted conclusions. Unfortunately the original paper was not available to the present authors.

The phenomenon of association–dissociation systems has been demonstrated with crystalline globulins from several types of seeds. Proteins from lupins (6, 7) and soybeans (8) have been studied and results have shown that the formation of components of specific sedimentation characteristics for each species is influenced by such factors as pH, time, and salt concentration. While the details vary considerably with proteins from different seeds, the general results are similar in nature.

The earlier work (1) left many questions unanswered and dealt with only one species of cucurbit. Smith and Greene (9) had stated that the amino

[1]Manuscript received August 24, 1959.
Contribution from the Department of Plant Science, University of Alberta, Edmonton, Alberta, with financial assistance from the National Research Council of Canada. Based on part of a thesis submitted to the Faculty of Graduate Studies as a requirement for the M.Sc. degree.

[2]Holder of Robert Tegler Research Scholarship (1955–56) and a National Research Council of Canada Studentship (1956–57).
[3]Dean of the Faculty of Graduate Studies, University of Alberta, Edmonton, Alberta.

acid composition of the crystalline globulins from the more distantly related species of the family showed distinct differences. Thus the globulins from pumpkin and squash (closely related species) yielded similar results but those from cucumber and watermelon (both belonging to different genera) could be distinguished from one another and from the proteins of squash and pumpkin.

Danielson (3) suggested that the ionic strengths of buffers used in the earlier study (1) were too low and that, if higher salt concentrations were used, the isoelectric range (pH 4.8–8.3) could be narrowed and isoelectric precipitation could be used to separate the fractions. While the earlier results were all opposed to this suggestion they were certainly not adequate to answer the criticism.

It was therefore decided to prepare crystalline globulins from seeds of the four species on which Smith and Greene had carried out their investigations. The results presented in this paper deal with the effects of time and pH over a wide range on the sedimentation behavior of these globulins.

Material and Methods

Supplies of seeds of the four cucurbits were obtained commercially in sufficient quantity to insure that all preparations of each were from a single lot. The species and varieties used were as follows: golden hubbard squash, *Cucurbita maxima*; sugar pie pumpkin, *Cucurbita pepo*; Chicago pickling cucumber, *Cucumis sativus*; Klondyke watermelon, *Citrullus vulgaris*.

Crude globulin was obtained from the seeds using methods outlined by Fuerst *et al.* (1) except that the ground defatted meal was not reduced further in the ball mill and extraction time was reduced from 3 to 2 hours. The extracted globulin was recrystallized three times, the crystals being taken up in two-thirds the original volume of the 10% sodium chloride solution. When the 10% solution was diluted to 2% by the addition of distilled water at 60° C the proteins were precipitated in crystals free from amorphous material.

Danielson (3) has criticized the use of ether extraction in the preparation of seed globulins because of the uncertainty of the effects of hot organic solvents. Since these seeds all contain considerable free fat and since such fat frequently adversely affects the base lines of sedimentation diagrams, it was considered advisable to extract it. It seems doubtful that any appreciable changes are brought about by ether in the Soxhlet apparatus used, since only relatively cool ether comes into contact with the protein, and ether extraction has been used as standard practice in the preparation of many proteins. Furthermore, a comparison was made of the sedimentation behavior of globulin prepared from extracted and non-extracted meals. The general nature of the patterns was the same.

Crystals were stored under the mother liquor at 4° C. Aliquots for use were removed by a pipette and the material transferred to a filter paper in a Büchner funnel. The mother liquor was removed by aspiration and the crystals rinsed lightly with distilled water with the precaution that little or no air passed through the crystal cake.

Two methods of dispersion were used. In the first, the crystal cake was dispersed directly in the buffer. In the second, the crystal cake was thoroughly broken up in distilled water and then the buffer added. The first method yielded clear dispersions only at pH values of 4.0 and below. Above pH 4, the dispersions were increasingly opalescent, but the opalescence could be greatly reduced by using the second method. Above the isoelectric range, however, opalescence occurred regardless of the method of dispersion. All solutions showing opalescence were largely cleared by low-speed centrifuging. The non-dispersed protein (usually very little) was no longer soluble in 10% sodium chloride and must therefore be considered to have been denatured.

Danielson (3) considered the methods used by Fuerst *et al.* (1) for dispersing the crystals to be unsatisfactory for dissociating systems. He suggested that the crystals should first be "dissolved" and then the buffer added, and the ionic strength altered. Essentially this method was used as one alternative in the present study although true dissolution was not obtained in water. At the higher pH values this method yielded higher concentrations of dispersed protein. There is no evidence, however, that either sedimentation or electrophoretic results were affected.

Nitrogen was determined using the micro-Kjeldahl method and pH was determined using the glass electrode.

Sedimentation analyses were carried out using a Spinco analytical ultracentrifuge at 250,000 times gravity. All sedimentation coefficients were corrected to the basis of water at 20° C and are reported as $S^0_{20,w}$.

Results

Solubility of Globulin

Satisfactory dispersions containing from 0.3 to 1.0% protein were prepared in all buffers of pH 4.0 and below, and in acetate buffers with concentrations of 0.04 *M* and lower and pH values of 4.0 to 4.8. From pH 4.8 to 8.2 the globulin was insoluble except in concentrated salt solutions such as the 10% sodium chloride (pH of 6.0) used as the original dispersing agent. Raising the pH of the sodium chloride solution had no effect but decreasing it below 5.8 resulted in the gradual development of opalescence, with precipitation of the protein at pH 4.8. Solubility was greatest at ionic strengths of 0.01 *M* to 0.03 *M* and decreased rapidly as ionic strength was increased. Concentrations of globulin usable for sedimentation analysis could be obtained in buffers of ionic strength above 0.1 *M* only at pH values below 4.0. Danielson's suggestion (3) that buffers of higher ionic strength should be used to increase solubility is therefore not feasible. The higher concentrations increased, rather than decreased, the isoelectric range.

Sedimentation Studies

Designation of Components

Throughout this investigation the various components could usually be readily identified by the sedimentation coefficients. While changes in sedimentation conditions, particularly in pH, caused changes in the S_{20} values obtained, there was usually little doubt as to which component was involved. For this

reason and for the sake of clarity and brevity the various components have been given the following designations:

(*a*) The lightest components found gave $S^0_{20,w}$ values of approximately 3.0 at pH 4.3. This agrees with the results of Fuerst *et al.* (1) and for each species this component is arbitrarily considered to be the monomer. It is designated S3, P3, C3, and W3 respectively for squash, pumpkin, cucumber, and watermelon proteins.

(*b*) All species yielded components with $S^0_{20,w}$ values of approximately 7.0 at pH 4.3 and (assuming it to be the same component (1)) of close to 8.0 above the isoelectric point. This component is designated S7, P7, C7, and W7.

(*c*) All species yielded components with $S^0_{20,w}$ values of approximately 12.0 and these are designated S12, P12, C12, and W12.

The actual mean sedimentation constants obtained are summarized in Table I.

TABLE I

Mean values for sedimentation constants of the components

		$S^0_{20,w}$			
Species	Component	At pH 4.3	No. of analyses	Above isoelectric point	No. of analyses
Squash	S3	3.0	8	3.5	1
	S7	6.8	8	7.9	6
	S12	11.8	4	12.3	6
Pumpkin	P3	3.1	7	3.5	2
	P7	6.7	7	8.0	5
	P12	12.7	12	12.7	5
Cucumber	C3	3.0	7	—	—
	C7	6.6	7	7.8	4
	C12	12.2	7	12.5	5
Watermelon	W3	3.0	9	3.2	1
	W7	6.4	8	7.9	3
	W12	13.2	7	13.0	5

Comparison of Components From the Four Species

Variations in $S^0_{20,w}$ values within species were as high as 10% and variations between species are therefore of doubtful significance. Despite this, the values obtained for W7 appeared to be consistently lower than those for S7 and P7 at pH 4.3 (Table I) and at all other pH values below 4.8.

The principal differences among the results obtained for the species are shown by the proportions of the components under varying conditions and by the effects on the dispersed protein of such factors as pH and time.

The diagrams in Fig. 1 illustrate the changes that occurred in dispersions of the globulins stored at 4° C. Preparations contained approximately 0.6% protein in 0.01 *M* acetate buffer at pH 4.3.

Fuerst *et al.* (1) showed that the proportion of S7 in squash globulin (their component II) gradually increased with the aging of the dispersion presumably

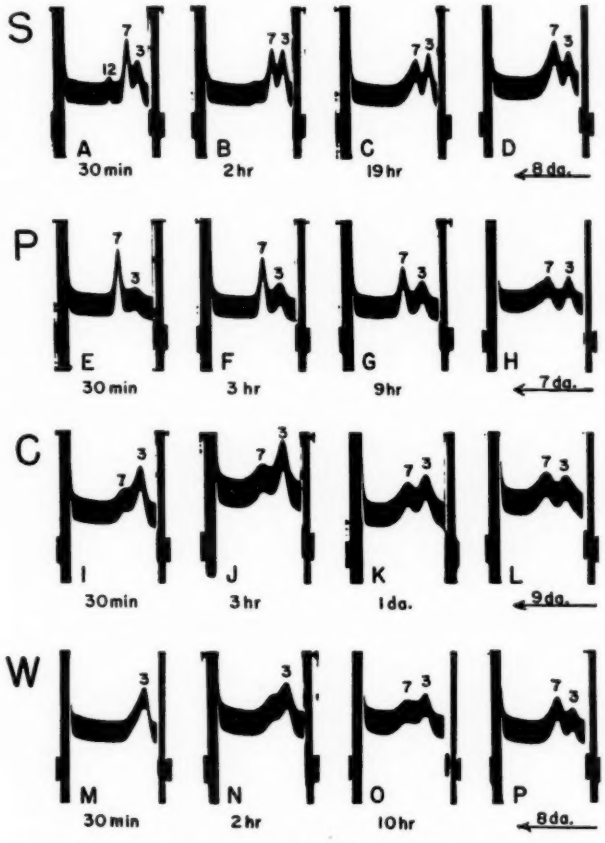

FIG. 1. Sedimentation diagrams obtained in 0.01 *M* sodium acetate, pH = 4.3. Time given with each diagram is the time after dispersion of the crystalline globulin. The diagrams presented were obtained approximately 40 minutes after the start of the run. A–D, squash; E–H, pumpkin; I–L, cucumber; M–P, watermelon.

at the expense of S3 (their component I). The results in diagrams A–D of Fig. 1 show that for some hours immediately following dispersion S3 increased at the expense of S7. The small but distinct component S12 present in the first diagram was not recorded by Fuerst and had completely disappeared 19 hours after dispersion, although there was a trace at 12 hours (diagram not shown). It seems probable that the process of dispersion was not immediately complete and that the S12 component broke down to the S7. The last diagram (D) in the first row confirms the results of Fuerst *et al.* since the S7 had definitely increased at the expense of the S3 at the end of 8 days.

No distinct P12 component appeared in pumpkin globulin at pH 4.3 (Fig. 1, diagrams E–H) although a faint suggestion occurs in two of the diagrams. In general the proportion of P7 decreased with time and was compensated by an

increase in P3. There was apparently not the distinct reversal at 7 days that occurred with the squash globulin.

The rapidity with which the monomer appeared on dispersion was greatest with watermelon globulin (Fig. 1, diagrams M–P) but also distinctly greater with cucumber protein than with that from either squash or pumpkin. The association of C3 and W3 to give C7 and W7 also took place faster than with the comparable components of either squash or pumpkin. For both, the association trend was very distinct and the C7 and W7 components occurred in larger proportions at 9 and 8 days respectively than at any earlier stage.

The diagrams in Fig. 2 present typical results obtained with the four proteins dispersed and studied at pH values of 3.6 (diagrams A–H) and 1.4 (diagrams I–P). These show close agreement among the results for the pro-

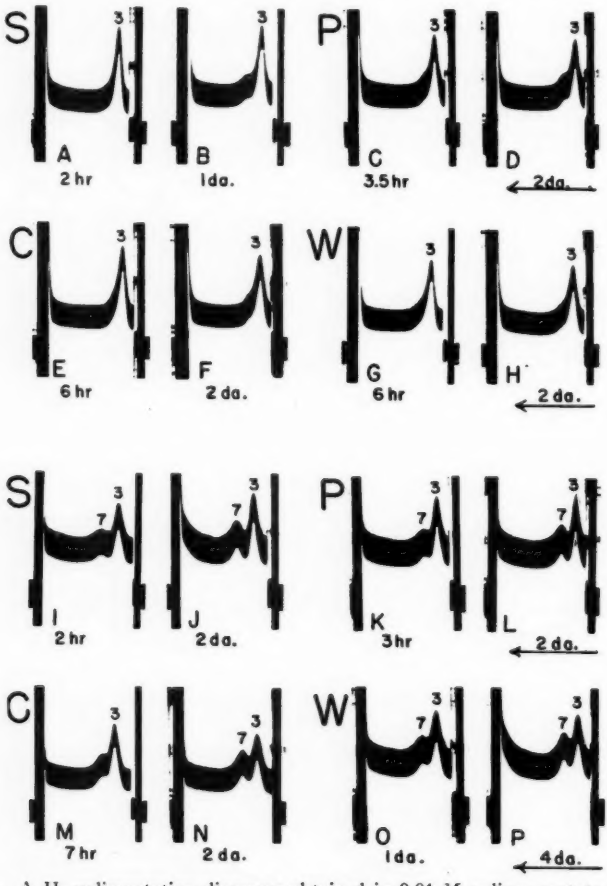

FIG. 2. A–H, sedimentation diagrams obtained in 0.01 *M* sodium acetate, pH = 3.6; I–P, diagrams obtained in 0.01 *M* glycine, pH = 1.4. Time given with each diagram is the time after dispersion of crystalline globulin.

teins from the four species and all results for each pH value can be discussed together. Sodium acetate buffer (0.01 *M*) was used at pH 3.6 and glycine–HCl buffer (0.01 *M*) was used at pH 1.4.

Most of the globulin existed in the monomer form at pH 3.6, although there were definite signs of the S7, P7, and C7 components after 2 or 3 days. The "7" components did not separate clearly but probably caused the skewing of the diagrams, particularly at the longer storage time. Only the dispersion of pumpkin globulin was stored for a prolonged period but after 23 days the diagram was essentially the same as that in Fig. 2D, the diagram for the same preparation after 2 days.

A series of analyses was carried out at pH 2.6, using glycine–HCl buffer (0.03 *M*). Diagrams are not presented since they essentially duplicate those obtained at pH 3.6 in sodium acetate buffer. A dispersion of squash globulin was stored for 25 days and the analysis, while indicating a small increase in S7, showed that over 80% of the globulin was still in the monomer form.

The analyses carried out at pH 1.4 show that there were no significant differences among the results for the four species. The heavier component occurred quite distinctly and the proportion of this component increased with time for each species. There is thus a definite reversal of the results as compared with those obtained at pH 3.6 or 2.6. At the moment, no satisfactory explanation can be offered for this difference. The high degree of acidity did not appear to adversely affect these proteins nor the actual analyses, although it may have been a factor in determining the sedimentation constants. This is discussed later.

The highest pH level below the isoelectric point at which an appreciable concentration of the globulin could be dispersed was pH 4.8. Solubility decreased abruptly above this point and insufficient protein was retained in solution to give satisfactory sedimentation diagrams. Comparable diagrams for the proteins of the four species at pH 4.6 to 4.8 are presented in Fig. 3.

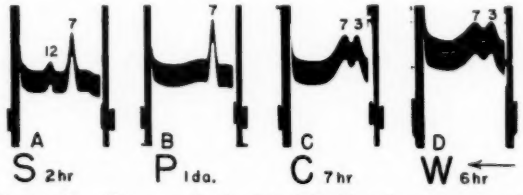

FIG. 3. Sedimentation diagrams obtained in 0.01 *M* sodium acetate, pH = 4.6–4.8.

The diagram for squash globulin at pH 4.6 shows the presence of the S12 component. Since the analysis was made 2 hours after preparation of the dispersion, it is possible that this component would have disappeared with time. It is significant that Fuerst *et al.* did not find this component below the isoelectric point. It was found, in the present study, only in relatively fresh preparations and it therefore appears probable that its presence was a measure of incomplete dispersion. The most important comparison obtained from the results illustrated in Fig. 3, diagrams A–D, is the distinctly different behavior

of the squash and pumpkin globulins on the one hand and the cucumber and watermelon preparations on the other. Components S3 and P3 had virtually disappeared from these preparations but C3 and W3 still made up an appreciable part of the total protein. On the other hand, the latter diagrams (C and D) are skewed to the left, indicating a tendency for heavier material to separate. Such separation did not occur during the analyses but the peaks became very diffuse by the end of the runs.

Figure 4 presents diagrams obtained at pH 5.8 to 6.0 in 10% sodium chloride. These agree with the single comparable diagram presented by Fuerst *et al.*

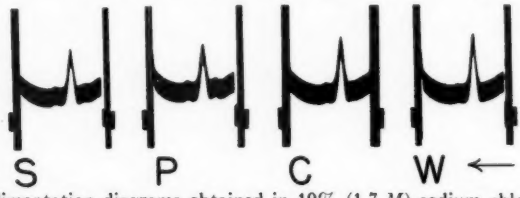

FIG. 4. Sedimentation diagrams obtained in 10% (1.7 *M*) sodium chloride, pH = 6.0, 1 day after dispersion.

and show similar results for the proteins from the four species. The main component had an $S^0_{20,w}$ value of 10.5 to 11.0 in each case and in each there are indications of one lighter and one heavier component with the exception that the lighter component appears to be absent from watermelon. All preparations are comparable in age, having been stored for 1 day. Fuerst *et al.* (1) reported the $S^0_{20,w}$ value as 8.2 for squash, but this was the uncorrected value. The uncorrected values obtained in the present study agree with that reported by Fuerst.

The final series of analyses was carried out on dispersions above the isoelectric point. The concentration of protein obtainable in buffers below pH 8.2 was negligible while a concentration of 0.18% protein was the highest obtained for watermelon globulin at 8.2. Solubility increased with increasing pH. The results for the protein from the four species analyzed at four levels of pH are presented in Fig. 5.

The light component, which has been termed the monomer, did not appear in any of these diagrams. The predominant component in all species at the lower pH values was the "12" but there were definite differences among species since S7 and P7 were distinct at pH 8.2 while C7 was only faintly discernible at pH 9.3 and W7 was absent at that level. Similarly, at pH 10.7, S7 and P7 were present in measurable quantities and there was a suggestion of S12 and P12, but an appreciable quantity of W12 was present. The diagram for cucumber at pH 10.7 (K) must be noted as it appeared that only C7 was present. This diagram does not fit into the series very well, however, because the component "12" was present at pH 11.0 for cucumber (L) and watermelon (P), and the behavior of the cucumber protein generally resembled that of watermelon rather than that of either squash or pumpkin. Watermelon globulin was not only the least soluble, but (at all pH levels) the proportion of the heavier fraction persisted more than with the other species.

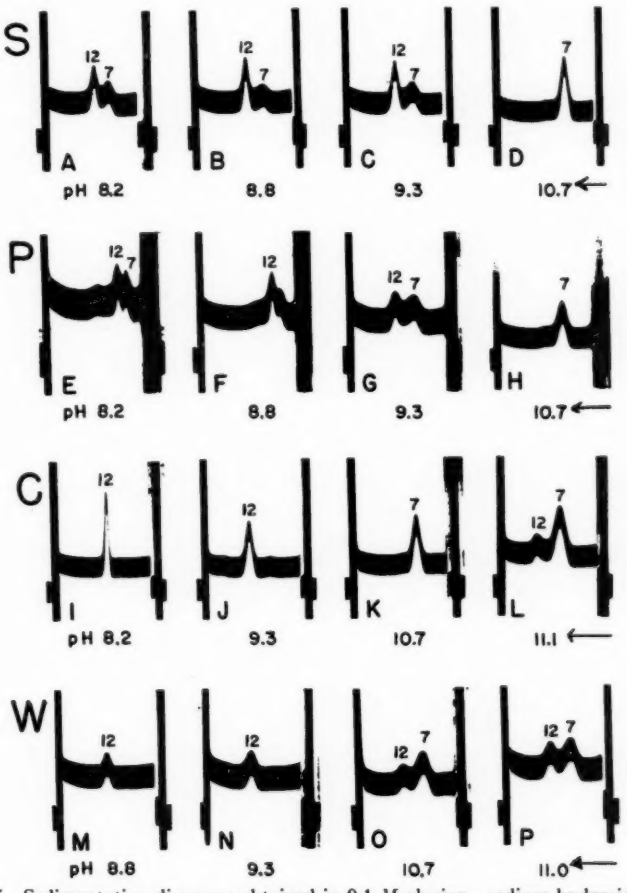

FIG. 5. Sedimentation diagrams obtained in 0.1 *M* glycine – sodium hydroxide, 1 day after dispersion.

It is recognized that working at high pH values is open to considerable criticism but a few of the results obtained above pH 11.0 appear to justify presentation. It was stated above that the monomer was absent from all diagrams in Fig. 5. Fuerst *et al.*, on the other hand, reported that at pH 11.6 a small amount of a light component, probably the "3" appeared.

In the present series of experiments sufficient of this light component was present in five runs to make possible reasonably accurate calculations. Results for two of these are shown in Fig. 6. The first three diagrams are from a single analysis of pumpkin globulin dispersed in 0.1 *M* sodium hydroxide. The exposures were taken 32 minutes apart. The important fact to note is that there is not more than a trace of the "7" fraction in this preparation but the globulin is nearly all in the form of the monomer ($S^0_{20,w} = 3.2$). The skewing

FIG. 6. Sedimentation diagrams obtained in 0.1 *M* sodium hydroxide, 1 day after dispersion. A–C, pumpkin globulin, three successive diagrams, 32 minutes apart, pH = 11.7; D, watermelon globulin, pH = 11.9.

toward the original boundary suggests that some partially hydrolyzed products of varying weights may have been present.

The last diagram (D) in Fig. 6 is for watermelon globulin at pH 11.9. The components are clearly the "7" and "3" with the latter decidedly the more prominent.

It appears therefore than the "3" component is the smallest encountered under any conditions, and that it occurred at both extremes of pH. Just how the other two main components are formed cannot be determined from the results obtained, but there seems to be no good reason to question the suggestion of Fuerst *et al.* that the "7" is the dimer and the "12" is made up of two of the "7" units. No really satisfactory explanation can be given for the $S^0_{20,w}$ values for the "dimer" being more than twice those for the "monomer". None of our studies have been directed specifically towards finding such an explanation.

Sedimentation Constants

Earlier in this paper it was pointed out that there was considerable variation in the values obtained for the sedimentation constants of the various components. Far too many individual results were obtained to report in detail, but it seems advisable to discuss a few of those that vary widely from the apparently normal values. This is particularly true because of the criticisms that have been levelled at the earlier studies (3). All $S^0_{20,w}$ values for analyses carried out above the isoelectric ranges of the globulins were reasonably constant regardless of the pH. The ranges for the four species were as follows: squash, 7.4–8.2 and 12.1–12.9; pumpkin, 7.7–8.2 and 12.1–13.1; cucumber, 7.5–8.1 and 12.3–12.9; watermelon, 7.8–7.9 and 12.4–13.4. There was no systematic variation.

There were greater variations below the isoelectric range. In general the $S^0_{20,w}$ values decreased as acidity increased but below pH 2.6 the trend was reversed. The results suggested that the low salt concentration of the buffers might be insufficient to remove the electrostatic effects. No exhaustive attempt has been made to study this factor but the work done suggests that this is not the only, or perhaps even the most important, factor involved. For example, the $S^0_{20,w}$ value for the lightest component of cucumber globulin at pH 3.0 was 1.5 in glycine buffer at 0.01 *M* while it was 1.7 in the same buffer at 0.1 *M*. Even more consistent results were obtained with watermelon globulin. It was analyzed at pH 3.0 in 0.01 *M* and 0.1 *M* glycine–HCl buffers and in

0.01 *M* formate buffer. The results were consistent at approximately $S^{0}_{20,w} = 1.5$. It is quite possible, however, that factors other than those already mentioned influenced the sedimentation rates.

Since this work was completed, Pedersen (10) has discussed the effects of pH and salts on the sedimentation of bovine serum albumin. Wide variations were found and Pedersen suggests that if comparisons of sedimentation values obtained in different laboratories are to be made the determinations should be made at the same pH and in similar salt solutions. The variation in results obtained in the present work are not necessarily explained by Pedersen's studies, but the variations he obtained were fully as great. A more detailed study of the cucurbit globulins would probably present a more complete explanation of the differences obtained.

Discussion

Most of the discussion section of the earlier paper (1) applies equally well to the present results.

Fuerst *et al.* concluded that the results obtained with squash globulin supported the theory that this crystalline globulin is a single protein that forms an association–dissociation system on dispersion. It is clear that the results of the present study confirm this and extend the conclusion to the crystalline globulin from pumpkin, cucumber, and watermelon. There is no evidence that each system included more than one protein species. Danielson's suggestion (3) that the results fit a two-component system cannot be accepted if, as we assume, he means two different protein species.

The "12" components of the present study are the equivalents of component IV in the earlier work, and the same stability over a wide pH range is established. Component III of that work did not occur in the present study and this was to be expected since it occurred only in very concentrated dispersions, none of which were analyzed in the present study.

Danielson's suggestion (3) that buffers of higher ionic strength should be effective in narrowing the isoelectric range was not supported, in fact the range was widened. The globulin was decidedly less soluble in higher buffer concentrations. There is little direct evidence that ionic strength seriously influenced the results obtained, even when 0.01 *M* buffers were used. The lower sedimentation constants obtained below pH 4.3 cannot be satisfactorily explained. While available direct evidence suggests that ionic strength was not the principal factor involved, it is inadequate to permit a final conclusion.

Species differences in solubility and in association–dissociation characteristics were clearly demonstrated, but differences in actual sedimentation coefficients were of doubtful significance. The close agreement between the results for squash and pumpkin globulin and the distinct differences between these and those for cucumber and watermelon proteins in general support the conclusions of Smith and Greene (9) although no work was done on amino acid composition. Watermelon globulin was definitely the most difficultly soluble, particularly above the isoelectric range. The species differences demonstrated in this study are not clearly enough defined to identify species and it is con-

sidered that the amount of work necessary to provide such identification is unjustified.

Electrophoretic analyses were carried out on many of the preparations discussed in this paper. The results are being prepared for publication.

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CONDUCTION BLOCK OF TERMINAL SOMATIC MOTOR FIBERS IN TICK PARALYSIS[1]

MAURICE F. MURNAGHAN

Abstract

In the perfused anterior tibial muscle of the tick-paralyzed dog acetylcholine in excess of the control value is not liberated on stimulation of the peroneal nerve; in the normal muscle 7 $\mu\mu$g of acetylcholine is liberated per nerve volley. The paralysis is evidently not due to defective synthesis of acetylcholine because acetylcholine is liberated in control and high-potassium perfusates, the choline acetylase activity and the acetylcholine content of lumbar ventral roots and peroneal nerves do not differ from that in normal dogs, and the tick-paralyzed muscle differs from that in the hemicholinium-treated animal in its response to a train of nerve pulses after previous tetanization. As somatic motor nerve fibers in the paralyzed dog have previously been shown to conduct a nerve impulse and the factors required for acetylcholine release at the nerve terminal apparently are not absent in the paralyzed animal, the mechanism of the paralysis is probably due to an inability of the nerve impulse to traverse the terminal presynaptic fibers. The 'lesion' evidently extends to the end of the presynaptic fiber, i.e. more distally than in botulism, because direct stimulation of the tick-paralyzed muscle fails to liberate acetylcholine.

Introduction

Tick paralysis, an acute ascending flaccid paralysis which may terminate in respiratory failure, occurs in North America, Australia, Europe, and South Africa (1). On the North American continent the disease has been confined largely to the northwestern region of the United States and southwestern region of Canada because the wood tick *Dermacentor andersoni* Stiles has been responsible for most of the cases. In recent years human cases due to the dog tick, *Dermacentor variabilis* Say, have been recorded in the southern and eastern United States (2). This disease merits attention because of the loss of livestock and human fatalities that it causes. To date, the only effective treatment is prompt removal of the tick.

Initial studies by the author (3, 4) showed that the paralysis produced in dogs by *Dermacentor andersoni* Stiles is apparently located at the neuromuscular junction, because an action potential was recorded from the peroneal nerve when the sixth lumbar ventral root was stimulated and the anterior tibial muscle contracted when stimulated directly but not when stimulated indirectly via the peroneal nerve. Further studies substantiated the hypothesis suggested in the earlier investigation that the mechanism is probably a failure of the nerve impulse to liberate acetylcholine at the nerve terminals as no end-plate potentials could be detected at located end-plate regions in the paralyzed muscle during nerve stimulation (5).

In this present study, which was carried out in the spring of 1958, the acetylcholine output of the perfused anterior tibial muscle during (*a*) nerve stimulation and (*b*) perfusion with a high-potassium Locke solution was compared in normal and paralyzed dogs. In addition the choline acetylase activity and

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Contribution from the Department of Pharmacology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario.

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acetylcholine content of lumbar ventral roots and peroneal nerves were measured to determine whether the inability of the nerve impulse to liberate acetylcholine in the paralyzed muscle is due to a defect in its synthesis.

Methods

The dog was kept in a cage which rested above a tray of detergent solution. Wild wood ticks, usually 20 females and 10 males, were applied to the clipped back of the neck of the dog approximately one hour after the animal had been sedated with morphine sulphate, 10 mg/kg intraperitoneally; the males were included as fertilization of the female evidently increases its feeding rate (6). The applied ticks were covered with a small dome-shaped wire capsule fastened to the surrounding hairs by cement; a small pledget of moist cotton was included to avoid desiccation of the ticks. To prevent the dog scratching off the neck capsule, an elastic adhesive bandage was wrapped around the chest of the dog; a hole cut in the bandage over the capsule permitted ventilation. As wild ticks are only procurable in the spring the study of this problem was consequently restricted to this period. Attempts to induce paralysis with cultured ticks have been unsuccessful to date.

Anesthesia was induced in dogs with ether or pentobarbital sodium, 30 mg/kg intraperitoneally, and maintained with 1% chloralose in hot saline intravenously. All branches of the anterior tibial artery were tied except those supplying the anterior tibial muscle, the extensor digitorum and peroneus longus muscles being removed during the dissection. The anterior tibial muscle was detached from its origin by dividing into six ligatured bundles and the peroneal nerve was freed for a distance of 4 inches back from the muscle. A polyethylene cannula was inserted into the lower end of the artery, the veins from the anterior tibial muscle cut and the upper end of the artery cut between ligatures. Heparinized saline was used to wash the blood out of the muscle which was transferred to a moist chamber maintained at 37° C and perfused with warm oxygenated Locke's solution (NaCl 9, KCl 0.42, $CaCl_2$ 0.24, $NaHCO_3$ 0.15, and dextrose 1 g per liter). Between cessation of the natural circulation and the beginning of the perfusion 10 to 15 minutes elapsed. After all traces of blood had been removed the perfusion fluid was replaced with 15 ml of Locke's solution containing eserine sulphate, 10^{-5} g/ml, which was continuously recycled, oxygenated, filtered, and maintained at 37° C. After 30 minutes the perfusate, in addition to a further 5 ml of fresh eserine–Locke used to wash out the apparatus, was collected; 15 ml of fresh eserine–Locke was added to the apparatus and a second perfusion period of 30 minutes began; the number of such perfusion periods varied between two and five in the different experiments. Perfusion pressure and flow varied between 50 and 150 mm Hg and 3 and 5 ml/minute respectively. The peroneal nerve was stimulated with square pulses, 1–4 volts, 0.2 millisecond, 5 per second; for direct muscle excitation 150-volt and 2-millisecond pulses were applied via needle electrodes.

In an early stage of the investigation the technique of 'through' perfusion as described by Emmelin and MacIntosh (7) for the cat's anterior tibial muscle

was tried. As the dog's muscle due to its relatively greater size required a greater perfusion flow to avoid anoxia, the concentration of the liberated acetylcholine was too small for measurement. Furthermore, if the acetylcholine output is decreased in the paralyzed muscle a larger number of nerve impulses would be required to liberate a sufficient amount of it for assay. Consequently recycling of a small volume of the perfusion fluid for an adequate period of time was adopted which permitted, at the same time, the application of a sufficiently large number of electrical pulses. Preliminary trials showed that acetylcholine did not hydrolyze appreciably even after 2 hours of perfusion under such conditions.

The morphinized–eserinized leech muscle (8) was developed at this stage as the assay object because the eviscerated cat could not be used due to the presence of adrenergic fibers in the peroneal nerve of the dog which liberate noradrenaline into the perfusate on electrical stimulation of this nerve. The perfusates were tested after dilution with distilled water (20 to 28 ml); the contractile substance was assumed to be acetylcholine if its response was blocked by *d*-tubocurarine (10^{-5} g/ml). Considerable edema of the perfused muscles developed as at the end of the experiment weights had increased by $90 \pm 11\%$ (M. $\pm$ S.E.M.). Dextran or polyvinylpyrrolidine were not incorporated in the perfusing fluid because the preparations that were available caused some contraction of the leech muscle; plasma was abandoned as a perfusion fluid because samples caused contraction of the leech muscle probably due to choline and/or potassium in haemolyzed specimens.

In the experiments where the muscle was perfused with high-potassium (23 mmolar) Locke solution the dog was injected 24 hours previously with 0.5 mg/kg reserpine intraperitoneally to reduce the noradrenaline content of the tissues; otherwise its possible release by the high-potassium Locke would mask the vasodilator effect of the acetylcholine when assayed on the eserinized–eviscerated cat. Acetylcholine standards containing an equivalent amount of potassium were used in the comparison. The high-potassium content of the Locke solution in these experiments precluded the use of the leech muscle as the assay object. Reserpine did not influence the development of paralysis.

The choline content in perfusates was determined by the method of Mentzer, Corteggiani, and Carayon-Gentil (9); the morphinized–eserinized leech muscle was used as the assay object.

For the determination of choline acetylase activity, acetone powders of the lower lumbar ventral roots and of the part of the peroneal nerve where it winds round the neck of the fibula were made in five normal and nine paralyzed dogs. The powders were made either immediately after the death of the dog or on the following day, the carcass being stored overnight in the refrigerator. The preparation of the acetone powders and the determination of choline acetylase activity followed that described by Hebb and Smallman (10) except that phosphotransacetylase (Worthington) and acetyl phosphate were used to convert coenzyme A into its acetyl form. The incubation mixture was as follows: coenzyme A 20 units, choline chloride 1.6 mg, KCl 12 mg, $MgCl_2.6H_2O$ 1 mg, acetyl phosphate 1.4 mg, *l*-cysteine 3.6 mg (previously neutralized

with *N* KOH), phosphotransacetylase (0.25% in 0.02 *N* $KHCO_3$) 0.25 mg, eserine sulphate 0.1 mg, 1 mg of ventral root acetone powder or 2 mg of peroneal nerve acetone powder in cysteine saline (6 mg cysteine/ml 0.9% NaCl), volume 1 ml.

The procedure for the determination of the acetylcholine content of the lower lumbar ventral roots and sciatic–peroneal nerve was similar to that described by MacIntosh and Perry (11) except that a high-speed blade homogenizer was used to disintegrate the tissue.

Paralyzed dogs anesthetized with chloralose were prepared for recording the mechanical or electromyographic response of the anterior tibial muscle. The muscle was stimulated either directly or through the peroneal nerve. In one experiment the anterior tibial muscle was prepared for close intra-arterial injection as described by Brown (12).

Results

Incidence of Tick Paralysis

In the 13 dogs on which ticks were applied paralysis was severe in 12 and moderate in 1; in severe paralysis there was no detectable contraction of the anterior tibial muscle during nerve stimulation.

The mean weight of the dogs was 5.48 ± 0.28 kg (S.E.M.). The corresponding values for the number of female ticks which were applied and became engorged were 19.1 ± 0.69 and 16.8 ± 0.89 respectively. In 12 of the dogs 6.42 ± 0.23 days were required for severe paralysis to develop and 7 days for moderate paralysis in the remaining dog.

The Acetylcholine Output of the Perfused Anterior Tibial Muscle during Nerve Stimulation

Figure 1 shows the results obtained in five normal and five severely paralyzed anterior tibial muscles. These results indicate that acetylcholine is apparently liberated from both the normal and paralyzed muscles during a control perfusion. However, while peroneal nerve stimulation increases the output of acetylcholine in the normal muscle, neither nerve nor direct muscle stimulation increases the acetylcholine output in the paralyzed muscle. The mean outputs of acetylcholine during stimulation in 10 perfusions in normal and in 8 perfusions in paralyzed muscles were 94.6 ± 7.3 and 30.5 ± 4.9 mμg respectively. The respective values in 7 control perfusions in normal and in 5 control perfusions in paralyzed muscles were 45 ± 8.2 and 43.2 ± 9.9 mμg. Although there is a significant difference ($P < 0.001$) between the control and stimulation values in normal muscles, no such difference ($P > 0.2$) exists between the control and stimulation values in paralyzed muscles.

The experiments apparently can be divided into two groups. The first consists of those in which the control output of acetylcholine evidently remains constant (experiments F and probably A, D, 1, 2, and 3); in the second group the control value falls throughout the experiment (experiments E and 4 and probably C and 5). In Fig. 1, where possible, the results of a normal muscle (A–F) are placed directly above those of a paralyzed one (1–5) where the latter's values are similar to the control values of the former. Experiments F and 3 are

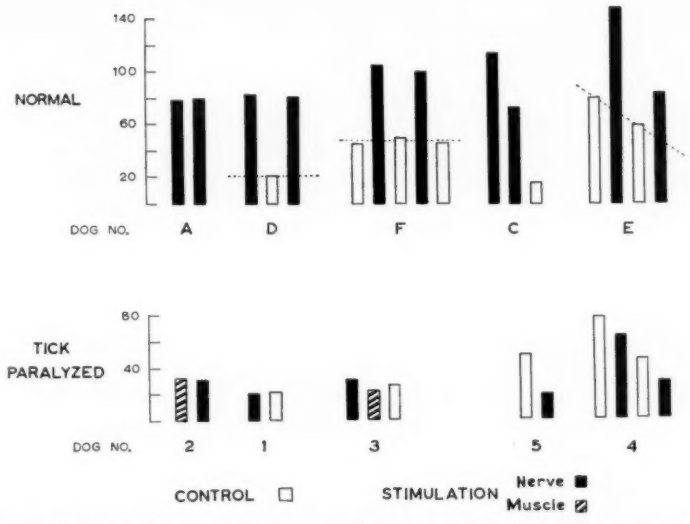

FIG. 1. Acetylcholine output, mμg, per 30-minute period of perfused anterior tibial muscle of dog.

exceptions in that the values of the paralyzed muscle are less than the control values of the normal muscle.

In Fig. 1 for experiments D, E, and F, interrupted lines have been drawn to indicate what the probable control values would have been during those perfusion periods when the nerve was stimulated. The mean calculated outputs of acetylcholine per nerve volley in these three normal perfused muscles were 7.3 ± 0.7 and 5.5 ± 0.86 μμg for the first and second stimulation periods respectively.

Acetylcholine Output of the Anterior Tibial Muscle during Perfusion with a High-Potassium Solution

Table I shows the results in a paralyzed and normal muscle. The first control perfusate in each experiment evidently contained a trace of nor-

TABLE I

Acetylcholine output of a normal and a paralyzed anterior tibial muscle perfused with normal and high-potassium Locke solution

Perfusion	Locke, mmolar K	Acetylcholine, mμg	
		Normal	Paralyzed
1	5.5	Pressor	Pressor
2	5.5	21	80
3	23	80	480
4	5.5	29	240
5	23	200	480

adrenaline which was absent in the second. The acetylcholine output increased in both muscles when perfused with Locke solution containing 23 mmolar

potassium. The greater output in the paralyzed muscle is probably of no significance due to variation among muscles as demonstrated in the previous perfusion experiments.

Choline Output of the Perfused Anterior Tibial Muscle

The mean outputs of choline (ion) per 30-minute perfusion period were 3.86 ± 0.5 and 3.74 ± 0.33 μg from paralyzed and normal muscle, respectively, corresponding to the respective concentrations in diluted perfusates of 0.13 ± 0.016 and 0.12 ± 0.011 μg/ml. These concentrations of choline were well below the threshold level (1–2 μg/ml) for the leech muscle and did not alter its sensitivity to acetylcholine.

The choline output in perfusates was independent of the following factors. (*a*) Nerve or muscle stimulation: As the choline output was relatively high in comparison with the amount of acetylcholine liberated by stimulation, there was no significant difference ($P = 0.5$) between choline outputs in control and stimulation perfusates. (*b*) The previous loss of choline from the muscle due to preceding perfusions: The mean choline outputs in perfusions 1, 2, 3, and 4 were 4.2 ± 0.6, 3.4 ± 0.3, 3.9 ± 0.7, and 3.8 ± 1.0 μg, respectively. These figures are derived from the pooled data of normal and paralyzed muscles as there was no significant difference between these two groups. No significant difference exists between the outputs of choline in the repeated perfusions during cumulative choline loss. The actual loss of choline from the muscle prior to each perfusion is unknown because the initial loss during the preliminary stage of the experiment, i.e. washing the blood out of the muscle, was not determined. Consequently the actual accumulated losses were greater than the summated outputs of previous perfusions. (*c*) Whether the control acetylcholine output was high or low and remained constant or diminished throughout an experiment: No correlation could be detected between choline output and control acetylcholine output in perfusions.

Choline Acetylase Activity and Acetylcholine Content in Nerve Fibers

Due to the low choline acetylase activity and acetylcholine content in muscle and the fact that these substances appear to be synthesized in the neuron and migrate down the axon (13, 14), they were estimated in the lower lumbar ventral roots and (sciatic–) peroneal nerves. The acetylcholine liberated per gram of acetone powder per hour for ventral roots was 15.6 ± 0.7 mg in five normal and 13.6 ± 1.8 in nine paralyzed dogs. The respective values for peroneal nerves were 1.3 ± 0.15 and 1.2 ± 0.2. The mean acetylcholine content of ventral roots was 8.4 ± 0.9 μg/g in four normal and 12.2 ± 3.3 in three paralyzed dogs. The respective values for the sciatic–peroneal nerves were 2.0 ± 0.3 and 2.5 ± 0.7. No significant difference existed between normal and paralyzed dogs either for choline acetylase activity or for acetylcholine content.

In the estimation of choline acetylase activity excess acetyl coenzyme A was supplied by its synthesis from coenzyme A and acetylphosphate with the aid of phosphotransacetylase. In order to exclude deficiency of this cofactor in the paralyzed animal, acetyl coenzyme A prepared either by enzymic or chemical synthesis (15) was (*a*) added to the Locke solution during perfusion of a para-

lyzed muscle, (*b*) injected intra-arterially into the anterior tibial muscle and intravenously in anesthetized paralyzed animals during recording of the mechanical response of the anterior tibial muscle when the exposed unligated sciatic nerve was stimulated, and (*c*) injected intravenously into unanesthetized paralyzed dogs. The approximate amounts of acetyl coenzyme A administered were in (*a*) 60 μg, in (*b*) and (*c*) 2 mg. In all cases no lessening in the paralysis occurred and in the unanesthetized dog the paralysis was still present 24 hours after the injection.

Miscellaneous Observations

In paralyzed anesthetized dogs in which the mechanical or electromyographic response of the anterior tibial muscle was recorded during peroneal nerve stimulation the following substances produced no apparent beneficial effect on neuromuscular transmission when administered intravenously. Atropine 1, choline 20, eserine 1, ephedrine 1, potassium chloride 20, calcium chloride 30, and hydrocortisone acetate 2 (mg/kg), and 100 ml of plasma from a normal dog administered after bleeding of the paralyzed dog. Induced asphyxia also failed to improve transmission.

In one moderately paralyzed dog during a train of seven pulses at three per second applied to the peroneal nerve at minute intervals no reduction of the fifth electromyogram with respect to the first occurred after a period of tetanization in the tick-paralyzed animal; in an animal treated with hemicholinium, which interferes with the synthesis of acetylcholine (16), the fifth response was markedly reduced with respect to the first (17).

Discussion

In an initial investigation in 1955–56 (4) the incidence of paralysis was low, there being only five severe and four moderate cases in 23 trials because the dog scratched off the ticks. In subsequent trials the use of the elastic adhesive bandage prevented this so that the incidence of paralysis increased, i.e. 6 dogs severely and 7 moderately paralyzed in 1957, and 12 and 1 respectively in 1958. Analysis of the results indicates that the application of an increasing number of female ticks so that more become engorged and using smaller dogs increases the incidence of severe paralysis, reduces the time of onset of the paralysis, and decreases the incidence of spontaneous recovery. Approximately one tick per kilogram evidently is required to produce paralysis and three per kilogram to produce a severe paralysis. While dogs, on a weight basis, exhibit about the same sensitivity as the other animal species tested to date, e.g. ground hogs, the human appears to be excessively sensitive, as one tick, particularly in children, commonly causes severe paralysis.

In the perfused anterior tibial muscle of the normal dog the acetylcholine output per nerve volley was 7.3 ± 0.7 $\mu\mu$g for the first stimulation period. Emmelin and MacIntosh (7) obtained a value of 4.3 ± 0.64 $\mu\mu$g when the anterior tibial muscle of the cat was perfused, but not recycled, with Locke's solution, plasma, or whole blood. While the difference between these values is significant ($P < 0.05$) the higher level in the dog may be due to difference in species or technique.

In recent perfusion studies on the superior cervical ganglion of the cat in which the Locke solution was not recycled, MacIntosh (16) has demonstrated that choline should be included in the Locke solution in order to maintain optimal acetylcholine synthesis and that carbon dioxide is required for its optimal release. It is possible that the acetylcholine values obtained in the normal muscles during stimulation in this investigation are below the optimal level because choline was not added to the perfusing fluid and pure oxygen was used for gassing it. However, choline and carbon dioxide liberated by the muscle accumulated in the perfusion fluid because it was recycled. The relatively constant loss of choline in repeated perfusates indicates that the muscles were probably not severely depleted of choline. Such constant losses in repeated perfusates, however, do not exclude a fall of muscle choline below a level for optimal acetylcholine synthesis. The greatest loss of choline probably occurred during the initial stage of washing blood from the muscle and the magnitude of this undetermined loss may have varied among individual muscles. Variation in this initial loss of choline and of other essential constituents and in the development of edema may account for the variation in the control acetylcholine output not only between muscles but also in an individual muscle.

Failure of synthesis of acetylcholine evidently is not responsible for the paralysis for the following reasons. (*a*) Acetylcholine undoubtedly was available at the motor nerve terminals in the paralyzed muscle because of its appearance in control perfusates and the large amounts liberated by a high-potassium Locke perfusion. (*b*) The choline acetylase activity and acetylcholine content of the lumbar ventral roots and the peroneal nerve were within normal limits. (*c*) The response of the anterior tibial muscle to a train of pulses after previous tetanization in the tick-paralyzed animal differed from that in the hemicholinium-treated animal.

Lack of factors believed necessary for the release of acetylcholine (e.g. the calcium ion) or its optimal release (e.g. carbon dioxide and the dialyzable chloroform-soluble fraction of plasma (16)) apparently is not responsible for the paralysis.

The site of the neuromuscular block in tick paralysis is evidently at the presynaptic terminal fibers of somatic motor axons. The lesion appears to extend distal to that produced by the botulinus toxin because in botulism, contrary to that in tick paralysis, direct stimulation of the paralyzed muscle liberates acetylcholine (18). In tick paralysis the nerve impulse evidently cannot traverse the presynaptic fibers to activate acetylcholine release due to a 'lesion' involving these fibers centripetally from and including the terminal end.

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SYNTHESIS OF DIPALMITOYL-L-α-GLYCEROPHOSPHORIC ACID DERIVATIVES, AS POSSIBLE INTERMEDIATES FOR SYNTHESIS OF GLYCERYLPHOSPHATIDES[1]

N. Z. STANACEV[2] AND M. KATES

Abstract

Using Verkade's method for the preparation of DL-α-phosphatidic acids, optically active dipalmitoyl-L-α-phosphatidic acid and its derivatives were prepared in good yield. The free dipalmitoyl-L-α-phosphatidic acid and its dimethyl ester were identical in melting point and specific rotation with the same compounds originally prepared by Baer using an alternate synthesis.

Phosphatidic acids were first discovered by Chibnall and Channon (1) in cabbage leaves, but it is now certain that the product obtained was largely the result of enzymatic degradation (2, 3). Similar products have been isolated from other biological material (see (4) for review of the literature). However, existence of phosphatidic acid per se in plants and animals has recently been established (5, 6, 7).

The synthesis of the racemic forms of α-phosphatidic acids has been attempted by a number of early investigators (see (4) for review of literature) but their methods will not be discussed here since they have not resulted in pure products. However, pure DL-α-phosphatidic acids and derivatives have been synthesized by Verkade *et al.* (8, 9, 10, 11, 12, 13), and the synthesis of L-α-phosphatidic acids and their methyl esters has been achieved by Baer *et al.* (4, 14).

Verkade's synthesis of the DL-compounds is based on condensation between DL-α-iododiglycerides and silver dibenzyl phosphate. The corresponding dibenzyl esters were thus obtained in 80 to 90% yields, and were converted to the free DL-α-phosphatidic acids by hydrogenolysis in the presence of palladium on charcoal.

Baer's synthesis of L-α-phosphatidic acids is based on phosphorylation of D-α,β-diglycerides with diphenyl–phosphoryl chloride. The diphenyl esters obtained were freed from phenyl groups by hydrogenolysis in the presence of platinum, and the L-α-phosphatidic acids were obtained in 75 to 82% yields.

Although both methods are very elegant, Verkade's method appeared to us to be more versatile. Thus, by treatment of an acetone solution of the dibenzyl ester with sodium iodide, the monosodium salt of the monobenzyl ester is readily obtained and can be converted to the corresponding silver salt. These salts should provide suitable starting materials for synthesis of other phosphatides, in particular the synthesis of phosphatidyl glycerol and phosphatidyl inositol. An additional advantage is that the preparation of diacyl iodoglycerides used in Verkade's method is easier to carry out than that of α,β-diglycerides used in Baer's procedure.

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[2]National Research Council Postdoctorate Fellow, 1958–1959.

For this reason we have investigated the use of Verkade's method for the preparation of optically active L-α-phosphatidic acid and its derivatives. Dipalmitoyl-L-α-iodo propylene glycol (prepared by acylation of L-α-iodo propylene glycol (15)) was phosphorylated using silver dibenzyl phosphate. The dibenzyl ester, obtained in a yield of 67%, was debenzylated, and the resulting free phosphatidic acid was found to have a melting point and a specific rotation identical with those reported for dipalmitoyl-L-α-glycerophosphoric acid by Baer and Maurukas (14). As a further check, the free acid was esterified with diazomethene and the resulting dimethyl ester was also found to have a melting point and a specific rotation identical with those of the dimethyl ester reported by Baer and Maurukas (14). In addition, the monosodium and monosilver salts of the monobenzyl ester have been prepared and their properties are described below.

Experiments are in progress to determine conditions for condensation of the sodium or silver salts of the monobenzyl ester of phosphatidic acid with various substituted inositol derivatives.

Experimental

Melting points (uncorrected) were taken in "Kimax" melting point capillary tubes in a heated stage melting point apparatus ("Cullati"–Zurich). All substances were dried *in vacuo* at room temperature over potassium hydroxide for 6 hours before analysis.

Dipalmitoyl-L-α-iodo Propylene Glycol (I)

L-α-Iodo propylene glycol (2.7 g, 13.5 mmoles) with m.p. 49° C and $[\alpha]_D$, −5.3° (2) was dissolved in 15 ml of dry, ethanol-free chloroform and 3.5 ml of dry quinoline was added. To this solution, at room temperature, 7.44 g of freshly distilled palmitoyl chloride in 15 ml of chloroform was added dropwise with stirring. The clear solution was kept in the dark for 6 days and was then diluted with 120 ml of dry ethyl ether. The ether layer was separated, washed once with 10 ml of 0.5 *N* sulphuric acid, three times with 10 ml of 5% sodium bicarbonate, and twice with water, and was then dried over anhydrous sodium sulphate. After evaporation of the solvent *in vacuo*, the residue was dissolved in 15 ml of dry ethyl ether, and absolute methanol was added until the solution was turbid. Upon cooling, 6.9 g (75.5% yield) of white crystals with m.p. 43–44° was obtained. After recrystallization from petroleum ether (30–60°) and methanol an analytically pure compound was obtained with m.p. 44–45° and $[\alpha]_D$, +3.3° in chloroform (*c*, 1.58). Reported m.p. for dipalmitoyl-DL-α-iodo propylene glycol is 46.5–47.5 (9). Anal. calc. for $C_{35}H_{67}O_4I$ (678.8): C, 61.92; H, 9.95; I, 18.69. Found: C, 61.89; H, 9.97; I, 19.06.

Dipalmitoyl-L-α-glycerophosphoric Acid Dibenzyl Ester (II)

Product (I) (3.7 g, 5.4 mmoles) was stirred under reflux in 50 ml of dry benzene in the dark with 2.3 g (6 mmoles) of silver dibenzyl phosphate (16) for 3.5 hours. The hot solution was filtered, the insoluble precipitate of silver iodide was washed with hot benzene, and the combined filtrates were evaporated to dryness *in vacuo*. The residue was recrystallized from 10 ml of

petroleum ether (30–60°), yielding 3.0 g (67%) of white crystals with m.p. 48.5–49.5° and $[\alpha]_D$, +4.2° in chloroform (*c*, 1.42). Reported m.p. for DL-compounds is 49–50° and an unstable modification melts about 45° (8). Anal. calc. for $C_{49}H_{87}O_8P$ (829.1): C, 70.98; H, 9.85; P, 3.74. Found: C, 70.76; H, 9.98; P, 3.86.

Dipalmitoyl-L-α-glycerophosphoric Acid Monobenzyl Ester Sodium Salt (III)

Product (II) (2.0 g, 2.4 mmoles) was refluxed with 540 mg (50% excess) of sodium iodide (dried at 120°) in 30 ml of dry acetone for 2.5 hours. After cooling in the icebox, the precipitate was filtered off, washed with dry, cold acetone, and recrystallized from about 50 ml of hot, dry acetone; 1.7 g (93%) of colorless microcrystalline sodium salt was obtained with m.p. 74–75° (sintering about 70°) and $[\alpha]_D$, +1.1° in chloroform (*c*, 1.75). Anal. calc. for $C_{42}H_{74}O_8PNa$ (761.0): C, 66.29; H, 9.80; P, 4.07; Na, 3.02. Found: C, 66.61; H, 9.84; P, 3.86; Na, 3.10.

Dipalmitoyl-L-α-glycerophosphoric Acid Monobenzyl Ester Silver Salt (IV)

Product (III) (900 mg, 1.2 mmoles) was dissolved in 75 ml of hot acetone and a solution of 205 mg (1.2 mmoles) of silver nitrate in 8 ml of water, diluted with 16 ml of acetone, was added. The silver salt precipitated immediately and was filtered, after cooling in the icebox overnight, to give 940 mg (94–95% yield) of colorless white microcrystals with m.p. 93–95° (sintering about 90°) and $[\alpha]_D$, +1.1° in chloroform (*c*, 1.55). Anal. calc. for $C_{42}H_{74}O_8PAg$ (845.9): C, 59.64; H, 8.82; P, 3.64; Ag, 12.75. Found: C, 59.45; H, 9.16; P, 3.49; Ag, 12.89.

Dipalmitoyl-L-α-glycerophosphoric Acid (V)

A suspension of product (II) (2.0 g, 2.4 mmoles) in 50 ml of absolute ethanol was shaken with 1 g of palladium on charcoal (10) in an atmosphere of hydrogen. In less than 10 minutes the theoretical amount of hydrogen was absorbed. The reaction mixture was warmed to 50° and filtered hot from the catalyst which was washed three times with 5 ml of hot chloroform. The combined filtrates were evaporated to dryness *in vacuo*, and the residue was recrystallized three times from ethyl acetate. The final product consisted of white crystals (820 mg, 52.8% yield) with a constant m.p. of 67.5° and $[\alpha]_D$, +4.0° in chloroform (*c*, 1.8). Reported values are: m.p., 70–71° (14); m.p., 65.5–66.5° (17); and $[\alpha]_D$, +4.0° (14). Anal. calc. for $C_{35}H_{69}O_8P$ (648.9): C, 64.78; H, 10.72; P, 4.78. Found: C, 64.62; H, 10.90; P, 4.85.

Dipalmitoyl-L-α-glycerophosphoric Acid Dimethyl Ester (VI)

Product (V) (150 mg, 0.23 mmole) was dissolved in 5 ml of ether and a solution of diazomethane in ether was added at room temperature until a yellow color persisted. After standing at room temperature for 12 hours the solution was evaporated to dryness *in vacuo* and the residue was recrystallized twice from methanol to give 145 mg (93.4%) of white crystals with m.p. 42–43° and $[\alpha]_D$, +2.0° in chloroform (*c*, 2.0). Reported m.p., 42.5–43.5°, and $[\alpha]_D$, +2.0° (14). Anal. calc. for $C_{37}H_{73}O_8P$ (680.0): C, 65.65; H, 10.87; P, 4.57%. Found: C, 65.78; H, 10.95; P, 4.54%.

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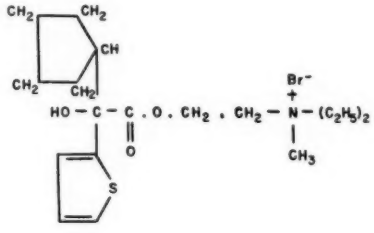
NOTES

PROTECTION OF HUMAN RED CELL CHOLINESTERASE AGAINST INHIBITION BY TABUN AND O,O-DIETHYL-S-2-DIETHYLAMINOETHYL PHOSPHOROTHIOLATE*

J. F. Scaife

Compounds which can slow the rate of attack of organophosphorus anticholinesterases upon the enzyme acetyl cholinesterase without appreciably affecting the activity of the enzyme would be desirable adjuvants to existing treatments for anticholinesterase poisoning. In vitro studies have shown that substrates (1–5), reversible inhibitors (1–4, 6), and other compounds (4–6, 7, 10), confer variable degrees of protection to cholinesterase against attack by irreversible inhibitors. A few of these compounds have afforded a measure of protection in vivo (7, 11, 12).

In this work a number of compounds, mostly antispasmodics or antihistaminics, were tested for their ability to protect human red cell cholinesterase against inhibition by tabun or O,O-diethyl-S-2-diethylaminoethyl phosphorothiolate (DSDP) in vitro. The compound WIN-4369,



was found to show some promise as a useful adjuvant to oximes in anticholinesterase poisoning.

Inhibition of cholinesterase (10% lysed human red cells) and its manometric estimation at 37° C, was performed as previously described (13). In the experiments with protectors both conventional and modified double-arm Warburg vessels were used. In the latter, one arm was Y-shaped so that it contained two separate sacs. One sac contained the aqueous inhibitor solution in 0.1–0.2 ml and the other the protector, 1 mg in 0.1 ml. In the conventional vessel the protector and inhibitor were mixed together for 45 minutes prior to the addition to the enzyme in the main vessel. Comparison of the results obtained using the two vessels then showed whether the protector produced any decomposition of the inhibitor which might otherwise be mistaken for protection.

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The amount of tabun added to each vessel was 0.2 ml of an aqueous solution containing 0.25×10^{-4} mm³ per ml. The amount of DSDP added was 0.1 ml of a solution containing 0.3×10^{-3} mm³ per ml. These amounts produced 73 and 63% inhibition of red cell cholinesterase respectively (I_i). The term I_{i+p} refers to the percentage inhibition of the enzyme produced by the mixture of inhibitor and protector, and I_p, the percentage inhibition due to the protector alone. The results with a number of compounds are set out in Table I. It will

TABLE I

Protection of human red cell cholinesterase against inhibition by tabun and DSDP at 37° C and pH 7.4

Protector (1 mg)	I_p*	I_{i+p}†		Protector (1 mg)	I_p*	I_{i+p}†	
		Tabun	DSDP			Tabun	DSDP
Antrenyl Br^-	0	56	9	Linadryl. HCl	6	75	69
Trasentin-6H. HCl	13	63	27	BW 53-322A. Cl^-	36	53	47
Trasentin. HCl	0	50	15	Artane. HCl	9	60	25
F.W.H. 101. Br^-	0	59	50	Lilly 01977. HCl	71	80	82
Benactyzine HCl	0	62	63	Chlorpromazine. HCl	56	69	65
Marsilid phosphate	2	71	65	Phenergan. HCl	28	65	41
Procaine. HCl	18	32	25	N,N-diethyl *p*-phenylene-diamine. HCl	7	47	11
Panparnit	24	42	36	N,N-diethyl benzene sulphonamide	8	47	18
Monocaine. HCl	28	69	52	Diethylamino ethanol	24	61	59
WIN-4369 Br^-	4	46	32	Diethylamino ethyl chloride. HCl	0	49	47
Benadryl. HCl	17	56	50	Diethylamino ethane thiol	42	70	50
Histol. HCl	67	72	70	Choline. Cl^-	15	41	41
SKF-525A.HCl	12	65	32				

NOTE: Inhibition by tabun alone 73%. Inhibition by DSDP alone 63%.
*I_p = percentage inhibition due to protector alone.
†I_{i+p} = percentage inhibition due to the mixture of protector and inhibitor.

be seen from Table I that the protective effect is not related to the affinity of the compounds for cholinesterase (I_p).

Experiments (given in detail elsewhere (14) in which the amount of inhibitor added to the enzyme was varied, while the amount of protector was kept constant, have shown that the protection was dependent upon the degree of inhibition produced by the inhibitor, decreasing as the ratio of inhibitor to protector increased. The action of the protector has been found to be to reduce the rate of attack of the inhibitor on the enzyme. It did not prevent the ultimate complete inhibition of the enzyme.

Under the experimental conditions and using the criterion outlined above none of the compounds tested caused any appreciable destruction of DSDP. However, the compound $HO.CH_2.CH_2.N.(C_2H_5)_2$ and, to a lesser degree, the methyl and isopropyl analogues were found to cause considerable decomposition of tabun in solution. The compound $HS.CH_2.CH_2.N.(C_2H_5)_2$ also showed some activity in this respect but was only about one-third as effective as the hydroxy analogue. Artane also showed some destructive action on tabun.

For various reasons such as solubility, toxicity, or cholinesterase inhibition a large number of the compounds screened in Table I can be considered unsuitable for in vivo testing. Some evaluation of the compound WIN-4369 has, however, been made in female albino mice. Used alone, this compound did not show any appreciable protective effect against DSDP. It did, however, show some ability to replace atropine as an adjuvant to the well-known oxime therapy with P-2-S (pyridine 2-aldoxime methane sulphonate). The results are given in Table II.

TABLE II

Protection of mice against DSDP by P-2-S atropine, and WIN-4369

	LD_{50}, μg per kg	Protection, LD_{50}'s
Therapy: P-2-S im., 50 mg per kg; Atropine sulphate im., 12.5 mg per kg; WIN-4369 im., 15.1 mg per kg — 15 sec after DSDP sc.		
DSDP	125	—
WIN-4369	280 mg/kg	—
DSDP + WIN-4369	200	1.6
DSDP + atropine	350	2.8
DSDP + P-2-S	960	7.7
DSDP + P-2-S + WIN-4369	3600	28.8
DSDP + P-2-S + atropine	6000	48
Prophylaxis: P-2-S oral, 200 mg per kg; WIN-4369 oral, 200 mg per kg; Atropine sulphate im., 12.5 mg per kg 15 sec after DSDP sc. — 15 min before DSDP sc.		
DSDP + P-2-S	330	2.6
DSDP + P-2-S + WIN-4369	850	6.8
DSDP + P-2-S + atropine	3200	25.6
P-2-S oral, 200 mg per kg; Atropine sulphate oral — 15 min before DSDP sc.		
DSDP + P-2-S + atropine, 12.5 mg per kg	1450	11.6
DSDP + P-2-S + atropine, 100 mg per kg	5700	45.6

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Defence Research Kingston Laboratory,
P.O. Box 123,
Kingston, Ontario.

AN ELUTER FOR PAPER CHROMATOGRAMS

M. Saffran* and D. F. Sharman

The eluter (Fig. 1) consists of a glass tube, with a female ground-glass joint on the top and a male joint on the bottom. A narrower glass tube, with a flared mouth, is sealed to the inner surface just above the bottom joint to make

*Fellow of the Foundations' Fund for Research in Psychiatry. Permanent address: Allan Memorial Institute of Psychiatry, McGill University, Montreal, Que.

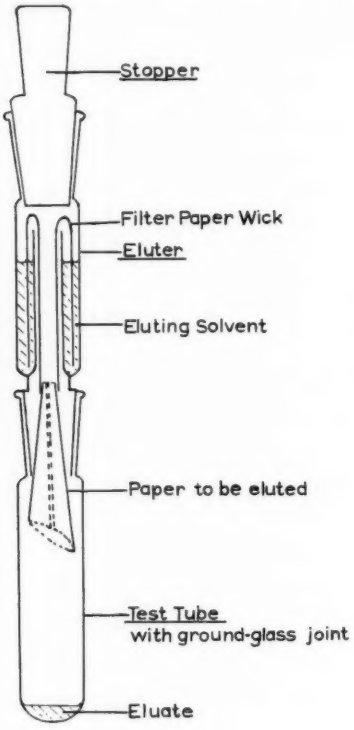
Stopper
Filter Paper Wick
Eluter
Eluting Solvent
Paper to be eluted
Test Tube
with ground-glass joint
Eluate

FIG. 1.

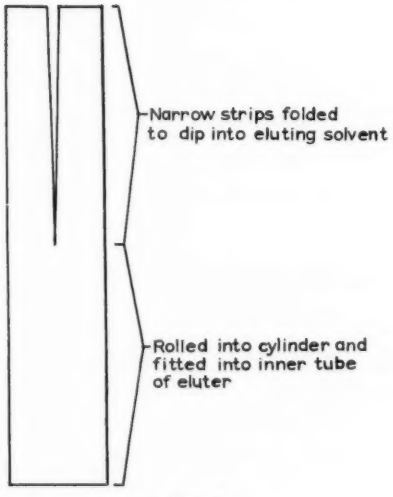
Narrow strips folded
to dip into eluting solvent
Rolled into cylinder and
fitted into inner tube
of eluter

FIG. 2.

a well for the eluting solvent. A filter-paper wick is prepared as in Fig. 2 and is fitted with one end in the solvent well and the other passing down the inner tube to its end. The wick must clear the top of the well to avoid syphoning of the eluting solvent. The paper to be eluted is shaped into a cylinder with a pointed tip and inserted in the bottom of the eluter until it just touches the bottom of the wick in the center tube. The paper cylinder is held in place by friction. The solvent is added with a Pasteur pipette to fill the well, the top of the eluter is stoppered, and a test tube with a female joint is fitted to the bottom (Fig. 1). The dimensions do not seem to be critical. Our eluters were fashioned with B14 Quickfit and Quartz joints (standard taper 14/26).

The solvent is drawn out of the well by the wick and brought to the paper to be eluted. The solvent then passes down the paper and drips into the collecting tube. In practice about 0.5 ml of eluant was usually collected in 1 hour. A faster flow can be achieved by substituting a hairpin-shaped glass capillary tube for the paper wick, but this modification has not been fully tested.

TABLE I
Recovery of chromatographed steroids with the eluter*

Steroid recovered	
Steroid chromatographed, 8.4 μg cortisol	Steroid chromatographed, 8.4 μg corticosterone
7.8	7.8
8.4	8.7
8.7	8.2
8.2	8.7
Mean recovery 8.3 μg or 99%	

*Eluting solvent, ethylacetate:methanol = 2:1; volume of eluate, about 0.5 ml; time of elution, about 1 hour.

The table illustrates the recovery of chromatographed corticosterone and cortisol using the eluter.

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Pharmacology Department,
University of Edinburgh,
Edinburgh, Scotland.

THERAPY OF ACUTE HYPOTHERMIA WITH SPECIAL REFERENCE TO UREA. A PRELIMINARY REPORT*

J. R. Beaton and J. Hunter

An excellent recent review of the current status of work in hypothermia has been prepared by Vandam and Burnap (1, 2). Andjus and Smith (3) have demonstrated that by enclosing a rat in a sealed container of 2-liter capacity placed in a refrigerator, the resultant fall in oxygen concentration and rise of carbon dioxide concentration permit successful cooling and resuscitation in the rat. It has been reported previously from this laboratory (4) that acetazoleamide (Diamox®) administered prior to cooling serves to prevent fatal

*D.R.M.L. Report No. 109–2, Project No. D50–93–10–72.

ventricular fibrillation commonly noted in untreated, cooled rats and permits successful resuscitation and survival. In adequate dosage, acetazoleamide inhibits the enzyme carbonic anhydrase (5) and is an effective diuretic (6).

It has been observed repeatedly that the hypothermic animal exhibits a marked lowering of blood pH (7, 8), and, indeed, becomes acidotic. We have routinely noted pH levels of the order of 6.8 in hypothermic rats. Nahas (9) has recently reported that the organic buffer 2-amino-2(hydroxymethyl)-1,3-propane diol (THAM) when administered intravenously to apneically oxygenated dogs restores arterial blood pH from acidic to normal values, presumably by buffering the CO_2. THAM has also been shown to increase urinary excretion of sodium, potassium, bicarbonate, and chloride (10).

Acetazoleamide and THAM share the common property of possessing a free $-NH_2$ group. Acetazoleamide has diuretic activity while such activity of THAM is uncertain. Urea is a naturally occurring substance which possesses the same characteristics and, further, is non-toxic in relatively large doses. For this reason, the efficacy of urea in permitting successful recovery and survival after cooling was studied.

As part of a long-term study on hypothermia in this laboratory, the comparative efficacies of acetazoleamide, THAM, and urea as therapeutic agents which might offer a measure of control in attaining recovery and survival from acute hypothermia were investigated.

Methods

Albino rats of the Wistar strain of both sexes weighing 200–250 g which were not fasted were used. Details of the cooling and rewarming techniques have been described previously (11). The rats were cooled under ice to a rectal temperature of 0 to 3° C which was usually accomplished within 55 to 60 minutes. Respiration ceased at a rectal temperature of approximately 14 to 15° C; no electrical activity indicative of an active heart and effective circulation existed below 7 to 10° C. On attaining the desired rectal temperature, the animals were immediately rewarmed in a constant-temperature water bath at 40° C together with forced ventilation using a 95% oxygen – 5% carbon dioxide mixture. The resuscitating procedure was routinely carried out for a 15-minute period on each animal at which time the rectal temperature exceeded 33° C. The animal was then placed in a ventilated plastic container maintained at 37° C for a further recovery period of 15 minutes. Thereafter the animals were maintained in a standard cage at room temperature with Fox Chow and water. The animals were observed for the ensuing 24 hours and interim deaths and the incidence of gross abnormalities, particularly limb paralysis, were noted.

Treatments, given by intraperitoneal injection in a volume of 3.0 ml were as follows: group 1, no treatment; group 2, saline;* group 3, saline containing 10 mg of acetazoleamide; group 4, saline containing 360 mg urea; group 5, an aqueous solution containing 5.25 mg NaCl, 1.11 mg KCl, and 110 mg THAM;

*0.85% sodium chloride in water.

group 6, saline containing 15 mg THAM adjusted to pH 7.4. THAM was given to group 5 in conjunction with KCl and NaCl since O'Connor *et al.* (12) used this dose in their studies with man. Urea was administered 8 minutes prior to cooling, this time lapse allowing elevation of blood urea to 114 ± 20 mg% from a normal level of 22 ± 3 mg%, ascertained by sacrificing a separate group of eight rats 8 minutes after urea administration. All other injections were given immediately prior to cooling.

The efficacy of treatment was determined by considering three criteria, viz., recovery after cooling, survival for 24 hours, and the condition of the animals after resuscitation and recovery.

Results and Discussion

The average cooling rates, the length of time the animals had a rectal temperature below 10° C, and the duration of forced ventilation and rewarming to initiate spontaneous breathing were essentially the same for all groups (Table I). The differences among groups appear in the number of animals alive 24 hours after cooling and resuscitation and in the incidence of complete or partial paralysis of the hind limbs.

TABLE I

Recovery and survival of rats after induction of acute hypothermia

Group No.	1	2	3	4	5	6
Treatment	None	Saline*	Urea, 360 mg	Acetazoleamide, 10 mg	THAM,† 110 mg	THAM,† 15 mg
No. of rats	12	12	12	12	10	12
No. resuscitated	9	11	12	12	10	11
No. surviving at 24 hours	3	8	11	10	2	10
No. normal‡ survivors at 24 hours	0	0	10	9	0	6
Final rectal temperature, °C	2.6±0.7	1.9±1.0	1.7±1.0	2.0±0.8	0.8±0.9	1.9±1.2
Initiation of breathing spontaneously, minutes	11±0.1	11±1.0	10±2.6	10±2.0	11±2.4	10±1.2
Rate of cooling below 15° C, °C/minute	0.7±0.2	0.7±0	0.8±0.1	0.7±0.1	0.7±0.2	0.7±0.2
Time below 10° C, minutes	16±6.6	16±4.9	15±3.3	20±4.2	19±4.0	16±5.5

*0.85% sodium chloride in water.
†2-Amino-2(hydroxymethyl)1,3-propane diol.
‡Not exhibiting paralysis or other apparent abnormalities.

It appears that saline per se has some beneficial effect in permitting an increased *survival* rate but the condition of the animals 24 hours after treatment was poor, marked paralysis of the hind limbs being present in all. As reported previously (4), acetazoleamide increased survival rate, the condition of the animals being satisfactory, with one exception, 24 hours after treatment. The results of urea administration were comparable to those of acetazoleamide administration. It is obvious from Table I that acetazoleamide and urea were far superior to no treatment and to saline treatment in permitting successful

recovery of rats to an apparently normal condition following hypothermia. No benefit resulted from the administration of THAM in group 5. Some benefit resulted from the administration of THAM at a lower dose of 15 mg in saline to group 6.

With regard to urea, we have observed in a separate study that the levels of endogenous blood urea prior to cooling and when the rectal temperature had reached 13° C were 22 ± 3 mg% (10 rats) and 22 ± 2 mg% (10 rats) respectively, i.e., no change in blood urea level resulted from this cooling. It is of interest that Andjus and Morel have reported that excretion of urine ceases in the cooling rat at approximately 18° C although increased urinary output can be observed at temperatures of 20 to 23° C (13). The possibility that active diuresis consequent upon urea administration occurs during and following rewarming cannot be overlooked and, indeed, this may well be a partial explanation of the beneficial effects of urea in acute hypothermia. Although quantitative measurements of water intake were not made, it was observed that urea-treated rats consumed abnormally large amounts of water in the 24-hour period following rewarming. It should be noted that Fisher *et al.* (14) have recently reported that urea administered intravenously to cats at a level of 1 mg/g body weight resulted in a marked lowering of carbonic anhydrase activity in blood although not in kidney. In this respect, urea is similar to acetazoleamide, although the latter compound also lowers carbonic anhydrase activity of kidney (5).

The apparent effectiveness of urea and acetazoleamide in preventing development of hind limb paralysis in rats rendered hypothermic and rewarmed is of considerable interest although a precise explanation is not available at the present time.

The present study is preliminary and a great deal of investigation is required to elucidate the best dose of these substances for administration, the time schedule for administration, and, most important, the mechanisms through which these substances bring about their beneficial effects in acute hypothermia. Further work is underway in this laboratory to clarify the underlying principles.

Acknowledgments

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Defence Research Medical Laboratories,
P.O. Box No. 62, Postal Station "K",
Toronto, Ontario.

Symposium on the Biological Effects of Radiation

This symposium was sponsored by the Canadian Federation of Biological Societies and was held in Hart House Theatre, the University of Toronto, on June 11, 1959, during the Annual Meeting of the Federation. The Federation Symposium Committee was headed by Professor J. V. Basmajian, who collaborated with the symposium Chairman, Professor E. A. Sellers, in arranging the program and editing the papers presented.

INTRODUCTION

E. A. Sellers

"The biological effects of ionizing radiation" was chosen as the subject of the Second Federation Symposium by the Symposium Committee of the Federation. This is a fitting choice because effects occurring soon after radiation will possibly, and effects occurring late after radiation will surely, present an ever-increasing problem to our civilization. A symposium on this subject lasting only 2 hours makes the selection of subject matter rather difficult because no other field in biology has expanded so rapidly in such a short time. Before the advent of nuclear weapons radiobiology was advancing at a measured pace in a few centers, largely because of its application in the therapy of cancer. The explosions at Hiroshima and Nagasaki produced results which have affected our outlook and our lives. Radiobiology is one of the fields which has experienced a mushroom growth.

With the advice of the participants of the symposium and others, particularly Dr. G. C. Butler and Dr. D. G. Baker, we have decided that the object of this symposium will be to review very briefly the broad field of effects of ionizing radiation—to present a bird's-eye view of the subject in an attempt to relate it to other fields with which members of the Federation may be more familiar. Of necessity, we have been forced to omit completely many important subjects.

In Canada the National Cancer Institute and its associated agencies, the Atomic Energy Corporation, the National Research Council, and the Defence Research Board have supported work, in this field, within and without their own establishments. Ten years ago only a few groups were actively engaged in radiobiological research. While Canada is not encouraging this type of research as much as many other countries—and there is need for extended activities—there are active groups in most provinces studying some aspect of this broad field. It would be possible to expand the aims of the symposium greatly without going outside this country for participants.

For my own participation as a speaker, as well as Chairman, I apologize. I must ask you to blame the Federation Committee for my dual role.

RADIATION, ITS PRIMARY INTERACTIONS[1]

G. F. WHITMORE

Abstract

A discussion is presented of some current thinking on the primary interactions of ionizing radiation with biological materials, including a brief discussion of ionization, excitation, the track phenomenon of charged particles, and the radiation chemistry of water. An attempt is made to correlate some of these phenomena with the variations in biological damage produced by various types of radiations incident on various biological systems and under varying conditions of oxygen tension.

If we are ever to understand fully radiation action then I believe we should be able to trace the development of damage from the time the radiation first interacts with the atoms and molecules which compose the irradiated material until the time the final damage manifests itself, whether this damage be cellular, somatic, or genetic in nature. Such an understanding will provide us with a partial answer as to why radiation is such an efficient producer of biological damage. My purpose is not to give you an extremely detailed picture of the processes whereby radiation energy is transformed to biological damage, but merely to provide a very brief outline of some of the facts that must be considered in any over-all theory of radiation action.

Perhaps the logical place to start this discussion is to look at the conditions which exist in a cell immediately after the passage of the charged particle through the cell. ("Immediately" in this context will refer to a time of perhaps 10^{-15} seconds.) Figure 1 is a diagrammatic representation of the conditions which exist.

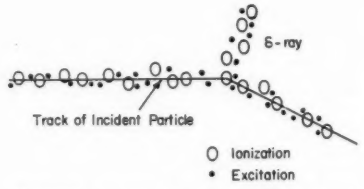

FIG. 1. Path of a charged particle in biological material.

In the figure the path of the incident charged particle is shown. For the moment we need not concern ourselves whether this incident particle is a neutron, a proton, an alpha particle, or an electron set in motion by an X ray. The charged particle loses its energy through the formation of only three types of primary products: the ionized and excited molecules depicted in the figure, and free electrons which are not shown. Each time an ionization occurs a free electron is removed and this electron may have enough energy to form

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Contribution from the Department of Medical Biophysics, University of Toronto, Toronto, Ontario, and the Ontario Cancer Institute, Toronto, Ontario. This paper was delivered at the Symposium on the Biological Effects of Radiation, Canadian Federation of Biological Societies, University of Toronto, Toronto, June 11, 1959.

a short spur track of its own, such as the one shown in the figure. Such a spur is known as a delta ray and both the original track and the delta ray are capable of causing biological damage. Figure 2 is a representation of the

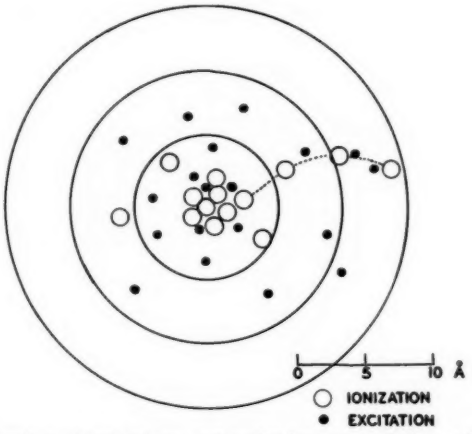

FIG. 2. Distribution of ionizations and excitations around the path of the incident particle.

distribution of ionizations around the track, as it would be seen by an observer looking along the path of the particle. It will be seen that the excitations and ionizations are restricted to a region immediately adjacent to the original track.

Although the ionizations and excitations which occur are scattered randomly along the length of the track, the average spacing is a very strong function of the speed and charge of the incident particle. The way in which the distribution of the charges along the track varies with the type of incident particle is shown in Fig. 3.

Particle	Mass	Charge	Track
X ray			
Electron	1/1843	−1	
Proton	1	+1	
Neutron	1	0	
Deuteron	2	+1	
Alpha	4	+2	

FIG. 3. A description of the six principal types of ionizing radiation together with representations of their track density. The length of track in all cases is 100 Å.

Here are listed the six principal types of radiation, the mass of each particle, and the charge on each particle. Beside each particle is a pictorial representation of a 100-Å length of track of that particle showing the number of ionizations and excitations which would be expected in that length of track. It will be noticed that an electron has a very sparse track whereas the neutron,

proton, deuteron, and alpha particles all have tracks of much greater density. In Fig. 3 there is no track shown for an X ray because an X ray incident upon a material loses its energy by one of three processes which result in the liberation of high-energy electrons. Since it is these electrons which do the biological damage, we can consider an X ray track to be roughly equivalent to an electron track.

As has been pointed out, the exact nature of the track is a function of the energy of the particle, and, in general, the track becomes more dense as the particle slows down but the general picture to carry in mind is that an electron has a relatively long but very sparse track, and an alpha particle has a very short but very dense track. The rate of energy loss along the track of a particle is usually referred to as the Linear Energy Transfer or L.E.T. of the particle. An electron or X ray has a low L.E.T. value while an alpha particle has an exceptionally high L.E.T. value.

In putting this much emphasis on the track effect, it must be pointed out that any complete theory of radiation action must allow for the reality of the track effect. That the track phenomenon is of great importance to our understanding of biology can best be illustrated by an example of a typical biological experiment in which the biological effects of two radically different types of radiation are compared.

When the anthers of the spiderwort, *Tradescantia bracteata*, are irradiated with a dose of 12 equivalent roentgens of alpha radiation, some 39% of the cells show chromosomal structural damage (1). To produce the same amount of damage using electrons would require a dose 10 to 20 times as large. When one remembers that an alpha particle loses energy at a rate 1000 times as fast as an electron this means that it may require 10,000 to 20,000 electrons to do the damage of one alpha particle.

In a further attempt to elucidate the reason for the great discrepancies which exist between the relative biological efficiencies of the various particles a great deal of work has been done not only in various biological systems but in various model chemical systems. For obvious reasons water is the material which has been most extensively studied and if we look briefly at the radiation chemistry of water, perhaps we can see some reason for the discrepancies in the radiation damage produced by alpha particles and electrons.

When radiation is incident upon pure water, it has been customary to assume that the primary products of irradiation were hydrogen and hydroxyl radicals, that is, $\dot{H}$ and $O\dot{H}$ formed according to the following reactions:

$$H_2O + \text{energy} \longrightarrow H_2O^+ + e^- \quad [1]$$

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + \dot{O}H \quad [2]$$

$$e^- + H_2O \longrightarrow \dot{H} + OH^- \quad [3]$$

There is some argument about the exact nature of these reactions but the large amount of experimental data which has been accumulated leaves little doubt as to the nature of the two primary products, the radicals $\dot{H}$ and $O\dot{H}$. Both of these substances are extremely active, one as a reducing agent, the other as an oxidizing agent, and it is very unlikely that any biological molecule

could resist attack by one or the other. This action of the radicals on biological molecules must account for a large part of the radiation damage produced by sparsely ionizing radiation. We could represent this by the equations

$$R + \dot{H} \longrightarrow R' \quad [4]$$
$$R + O\dot{H} \longrightarrow R'' \quad [5]$$

where R is a biologically active molecule and R′ and R″ are inactive forms of the molecule.

However, in addition to these reactions with dissolved biological molecules, the radicals themselves undergo a number of competing interactions including the following:

$$\dot{H} + O\dot{H} \longrightarrow H_2O \quad [6]$$
$$\dot{H} + \dot{H} \longrightarrow H_2 \quad [7]$$
$$O\dot{H} + O\dot{H} \longrightarrow H_2O_2 \quad [8]$$

Since we believe that Ḣ and OḢ are derived from the primary excitations or ionizations along the track of the incident particle, we would expect the local concentration of Ḣ and OḢ to be proportional to the concentration of these events and to be much greater along the tracks of very densely ionizing particles such as protons and alpha particles. In fact, a simple calculation reveals that along the track of a densely ionizing alpha particle there may be localized regions in which the concentration of Ḣ and OḢ might be as high as 1 *M* even though their average concentration throughout the cell might be as low as 1/100 μmolar.

In regions of high concentration of Ḣ and OḢ it might be expected that reactions [6], [7], and [8] would predominate over reactions [4] and [5], that is, one would expect a very large production of hydrogen gas and H_2O_2. As will be seen, this assumption has been adequately confirmed by experiment. Also, it might be expected, because of the very high concentration of OḢ along the track, that the production of H_2O_2 would be almost independent of the presence or absence of dissolved solutes in the water. This assumption has also been adequately confirmed by Ebert(2), who observed the same yield of H_2O_2 in pure water irradiated with alpha particles as in 20% ethyl alcohol even though ethyl alcohol has a very high affinity for OḢ radicals. Along the tracks of sparsely ionizing particles, the chance of two radicals reacting would be very much reduced and would allow the Ḣ and OḢ radicals to diffuse away and react with biological molecules according to reactions [4] and [5], resulting in the production of biologically inactive molecules.

In the experiment in which the *Tradescantia* were irradiated and the number of chromosome breaks observed, it was clear that alpha particles were very much more efficient than electrons as producers of chromosome breaks. However, Lea and Salaman in 1942(3) reported an experiment in which they irradiated vaccinia virus with both alpha particles and high-energy X rays and measured the relative efficiency using inactivation of the virus as a criterion of biological damage. In these experiments, it was found that if the X rays had an efficiency equal to unity, the efficiency of the alpha particles was only

0.38. Here, then, are two cases, one in which alpha particles are more efficient and one in which alpha particles are less efficient than electrons as producers of biological damage.

Table I gives a very brief summary of certain other experiments comparing X rays, electrons, neutrons, and alpha particles on the basis of their relative

TABLE I

Irradiated object	Effect studied	Radiations	L.E.T., kev/μ	Relative effectiveness
Water	Production of H_2O_2	X	3	0
		α	120	1.0
Carboxypeptidase	Inactivation	X	1.2	1.0
		α	120	0.05
Virus, vaccinia	Inactivation	γ	0.35	1.0
		X	4.7	0.77
		α	110.0	0.38
Virus, phage C16	Inactivation	X	2.5	1.0
		α	120	0.18
Yeast cells	Inactivation	X	2.5	1.0
		α	190	3.4
Tumors in vivo mouse lymphoma	Production of chromosome abnormalities	X	2.5	1.0
		Neutrons	8.5	2.3–3.5

biological efficiency. This is by no means a complete listing of experiments of this type (4) and the only purpose in showing it is to establish one or two rather general trends.

This table illustrates several significant factors. As predicted, when water is irradiated, the yield of hydrogen peroxide is very much greater with alpha particles than with X radiation. For the next three systems in the table, the enzyme carboxypeptidase and two viruses all in aqueous suspension, the inactivation per unit dose decreased with the increasing L.E.T. However, for the last two systems, the inactivation of yeast cells and the production of chromosome abnormalities, the trend is quite similar to that for the *Tradescantia* in that the efficiency of the radiation increased with an increase in L.E.T.

The question which now arises is whether any or all of these findings can be explained using the knowledge of track formation and radical chemistry already discussed and, if so, how?

In attempting an explanation, some simplified diagrams will be used (Fig. 4). In the figure are shown two systems, a very small virus irradiated in aqueous suspension and a relatively large and organized structure, which we might think of as a chromosome or similar biological entity, and we will make use of two types of radiation, a sparsely ionizing particle and a densely ionizing particle.

In both cases, it is assumed that the total number of ionizations produced is a constant. In the case of the virus, we are interested in the number of viruses inactivated, and in the case of the chromosomes, we are interested in the number of chromosome breaks. In the case of the virus, we assume that any ionization within the virus and any radical which strikes the virus will

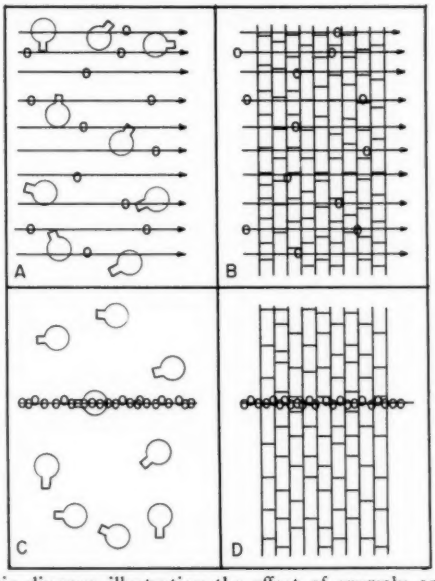

FIG. 4. Schematic diagram illustrating the effect of sparsely and densely ionizing radiation on two types of biological systems.

cause inactivation. For the chromosome, we assume that it takes several closely linked interactions to cause a break. In the virus case, it can be seen that two viruses suffer direct hits in A whereas only one is hit in B. Also, in A the radicals tend to diffuse until they either strike a virus and inactivate it or strike another radical and recombine harmlessly, and so this diffusion of radicals gives rise to further inactivation. In case B, most of the radicals rapidly react with other radicals to give a dense "squirt" of hydrogen peroxide and hydrogen gas; but, since there is only one virus in the region of high concentration of H_2O_2, this does not give rise to further inactivations and, in diffusing out to strike other viruses, the hydrogen peroxide concentration is reduced to an ineffectual level. Therefore, in accordance with experimental findings, we would expect much more virus inactivation with sparsely ionizing particles.

In the case of the chromosome, where the object is to find breaks, more small breaks occur with sparsely ionizing radiation and from the diffusion of radicals. However, in most cases, because of the large number of bonding structures in the chromosome, the reaction of one ionization in a region of chromosome does not produce a visible break, although it might conceivably produce a mutation. The ionizations are too widely spread for any significant amount of co-operation. However, with the densely ionizing particle, a region exists in which many ionizations have occurred and a region in which there is a strong concentration of hydrogen peroxide. The combination here is likely to produce a break and, therefore, in this case, the densely ionizing particle is the most efficient.

Further evidence concerning the role of radicals and other reactive chemical species in radiobiology has come from a study of the way in which the biological damage produced by a given dose of radiation is dependent on the oxygen concentration in the biological system at the time of irradiation (5, 6). The general trend of the results of such experiments is indicated in Fig. 5. It shows

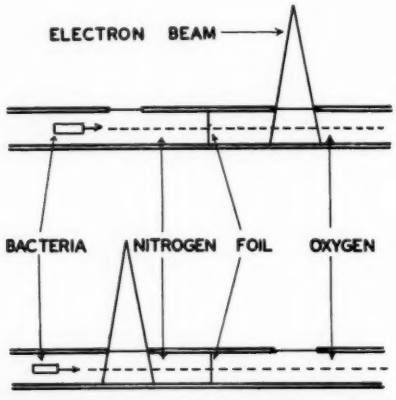

FIG. 5. Effect of oxygen and nitrogen on the radiation sensitivity of Ehrlich ascites cells to X rays and neutrons.

the inactivation of Ehrlich ascites tumor cells irradiated with X rays and neutrons in atmospheres of nitrogen and oxygen (6). With both types of radiation, the cells were more sensitive in oxygen than in nitrogen but with X rays the difference in sensitivity was greater than with neutrons.

While this is only an isolated experiment, it is a very general observation that the presence of oxygen enhances the production of radiation damage and that the enhancement is more pronounced with X rays than with neutrons or other densely ionizing particles.

The question now arises as to whether the oxygen effect is due to some physiological changes in the cell, due perhaps to an altered metabolism, or whether the oxygen exerts its effects on the primary interactions between radiation and the cellular material. Some recent and very elegant experiments by Howard-Flanders and Moore (7) have demonstrated almost beyond any doubt that the effect of oxygen is due to a basic interaction with the primary products of irradiation rather than an altered physiological state of the cell. Figure 6 is a schematic representation of the experimental method. The setup consisted of a twin-chambered radiation device with the two chambers separated by a very thin foil. The atmosphere in one chamber was pure oxygen and in the other pure nitrogen. Each chamber was provided with a radiation port so that the radiation could be incident on either chamber. The bacteria to be irradiated were mounted on a fast-moving carriage which could penetrate the thin foil separating the compartments. The time of transit of the carriage from the electron beam to the foil was about 10 milliseconds. In the first

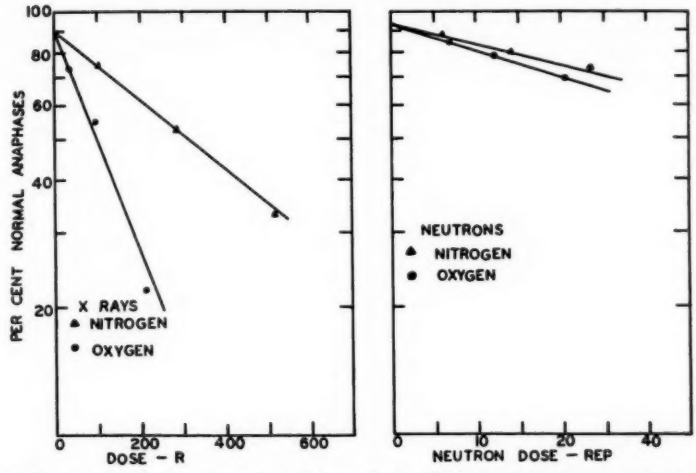

FIG. 6. Schematic representation of the radiation device used by Howard-Flanders and Moore.

experiment, bacteria stored in nitrogen were passed through the foil into an atmosphere of oxygen and then irradiated 10 milliseconds after contact with the oxygen. In the second case, the bacteria were irradiated in pure nitrogen then passed into oxygen within 10 milliseconds after irradiation. In the first case, the bacteria showed the full oxygen sensitivity even after only 10 milliseconds exposure; in the second case, the bacteria showed the full anaerobic sensitivity even though they passed into oxygen 10 milliseconds after irradiation.

The results of this experiment indicate that oxygen need be present only at the time of irradiation, which would seem to rule out any physiological changes, and that the oxygen must be involved in some early radiation effect which is complete within 10 milliseconds after irradiation.

There are several postulates in the literature attempting to explain the oxygen effect. The latest, that due to Howard-Flanders and Moore, assumes that radiation produces carbon radicals which react with oxygen to form peroxides. A second hypothesis, usually used for aqueous systems, assumes the reactions

$$\dot{H} + O_2 \longrightarrow H\dot{O}_2 \text{ (the hydronium radical)}$$
$$HO_2 + 3H \longrightarrow 2H_2O$$

in which a single reducing radical $\dot{H}$ has been transformed into a triply oxidizing radical which then removes three hydrogens from various biological molecules. If oxidizing and reducing agents are equally effective as producers of biological damage then this might give us as much as threefold increase in sensitivity. For densely ionizing particles, the oxygen effect is probably reduced because the oxygen cannot diffuse fast enough to react with hydrogen radicals before they react with one another.

In review, it will be realized that the work that has been discussed has not taken us very far along the road toward a complete understanding of radiation damage. It has shown the nature of the primary products of radiation, excitations and ionizations, which are localized along the length of the track of the incident particle; also these ionizations may give rise to radicals and some of the possible interactions of these radicals. In addition the role of oxygen in radiation damage has been noted.

While we can be satisfied with the observations of all these phenomena, it is obvious that most of the explanations have been hedged with assumptions and the conclusion must be that there remains a very great deal of work to be done before we will fully understand these basic processes of radiation action, let alone the ways in which they must be magnified to account for the incredible efficiency of radiation as a producer of biological damage.

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RADIATION, THE CELLULAR APPROACH[1]

GORDON M. CLARK AND DONALD G. BAKER

Abstract

The basic biological action of radiation must initially involve particular cells. The sensitivity of various cells differs but some of the differences may be due to a dose-rate effect. When cells are irradiated with doses in the lethal range, inhibition of cell division occurs. A notable feature of mitotic inhibition is that it depends not only on dose but on dose rate. Chromosome breaks produced in cells may be a visible manifestation of an interruption in chemical synthesis produced by the passage of ionizing particles. The effect of oxygen is apparently of primary importance in biological changes produced by irradiation. Almost all are decreased in its absence. The current trend in research on the cellular level is towards determining the more obscure injuries which cannot be seen under the microscope.

Introduction

Radiation biologists are concerned chiefly with how rays of wave lengths outside the visible spectrum can alter a cell, a tissue, or an organism. Radiobiologic action begins with changes in the atomic structure of cellular constituents. If the component modified is a critical one, radiobiological action is

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Contribution from the Department of Zoology, University of Toronto, Toronto, Ontario, and the Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario. This paper was delivered at the Symposium on the Biological Effects of Radiation, Canadian Federation of Biological Societies, University of Toronto, Toronto, June 11, 1959.

reflected in an altered cell behavior which has an influence on the organism and the population as a whole.

The basic biological action of radiation must initially involve particular cells. To obtain fundamental information concerning the biological effect of irradiation we must study its effects on isolated cells or cell colonies. It is at the cellular level where we might expect to observe the most direct expression of the radiation response.

Cytological Effects

(a) Variation in Sensitivity of Various Cells

The most obvious effect of irradiation on cells is death. One of the remarkable features of ionizing radiation is the difference in sensitivity of different cells and organisms. Most mammals are killed with dosages of 200 to 1000 rads, whereas single-celled animals, such as the ciliated protozoan *Tetrahymena pyriformis*, are killed by dosages ranging from 300 to 600 kilorads (1). The sensitivity of isolated mammalian cells varies also; while lymphocytes may be killed by 300 rads some cells from the skin may require 5000 rads (2). No convincing reasons have yet been advanced to explain why there are these tremendous variations between different cells in their response to radiation.

In recent years we have been studying the effects of X irradiation on the germination of buckwheat seed (*Fagopyrum esculentum*, variety silver hull) with chromosome numbers of $2n = 16$ and $4n = 32$. We have confirmed (3) that there is a decreasing sensitivity to radiation with increasing chromosome number. Of particular interest is the fact that the total dose administered to the seed is not as important as the rate at which the irradiation is given, i.e. the dose rate. Maximal effects as indicated by a decreased percentage germination of the seed were seen at certain dose rates. The variation in the response of a variety of cells to X irradiation may be explained in part by variations in dose rates. The response following irradiation may vary quantitatively, and perhaps qualitatively, in cells receiving the same total dose but at different dose rates.

(b) Retardation of Cell Division

The most obvious detectable change when cells are irradiated with doses in the lethal range is a decrease in rate of cell division for a limited period of time and consequently a retardation of growth. Studies with chick fibroblasts in tissue culture have shown that the sensitivity of the cell varies and depends on the particular stages of the mitotic cycle at the time of irradiation. Mitosis does not stop immediately after irradiation but goes on for some time. This is followed by a period when there is no division. Cells irradiated during division (metaphase to telophase) complete the mitotic cycle with little or no delay and usually survive to undergo successfully further division (4, 5, 6).

Cells irradiated at interphase and early prophase will cease dividing. There are exceptions, however, and in the case of grasshopper neuroblast cells the critical period occurs just before the disappearance of the nuclear membrane. Irradiation of a cell before it has reached this period usually causes it to stop or even revert to an earlier stage of prophase (7, 8). In Fig. 1 the effect of

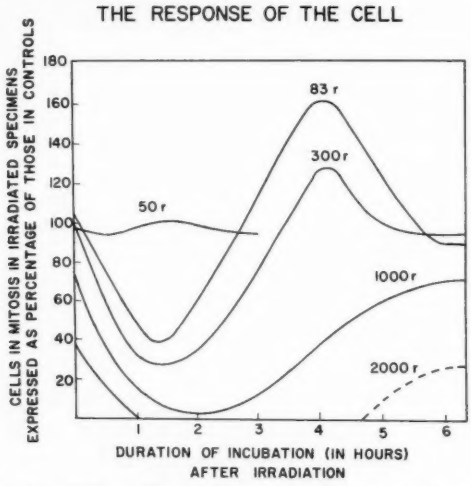

FIG. 1. Effect of increasing doses of X rays on the rate of the cell division of cells grown in tissue culture (chick fibroblasts). Up to 300 rads there is only a temporary depression and after the compensatory wave the culture returns to normal. With 1000 and 2000 rads, mitosis is completely suppressed for a time and the culture does not recover completely (after Canti and Spear, 1929).

different doses of X rays on chick fibroblast cells in tissue culture is shown. Immediately after irradiation with small doses (50 rads), the number of dividing cells remains unchanged, but falls and reaches a minimum after several hours. Following this there is an increase in the mitotic activity and a compensatory wave of cell division greater than normal, after which normal mitotic activity resumes. The temporary increase in the number of cells in division in the second part of the S-curve is not due to an enhancement of cell growth by irradiation, but is the result of the entry into division of temporarily arrested cells and of cells not affected by the radiation received.

A notable feature of mitotic inhibition is that it depends not only on the dose, but on the dose rate (9). It can be seen from Fig. 2 that when the dose rate is low the duration of mitotic inhibition in *Tradescantia* microspores is short and at very low dose rates disappears entirely. Possibly there is a dose rate at which for a given dose, the mitotic inhibition is greatest. Work now in progress in our laboratory indicates that this is true in the case of buckwheat. Such a possibility suggests that there is a short period in the mitotic cycle when the cell is most susceptible to damage. It is during this period that one observes maximal mitotic inhibition. In various mammalian tissues (e.g. mice) the mitotic times are similar; the variable appears to be the intermitotic time (10). This would perhaps explain the wide variations in the response of various tissues to ionizing radiation.

(c) Chromosome Breakage—Direct and Indirect Theories

As early as 1903 it was reported that irradiation caused fragmentation of chromosomes and a maldistribution at cell division. It was also postulated

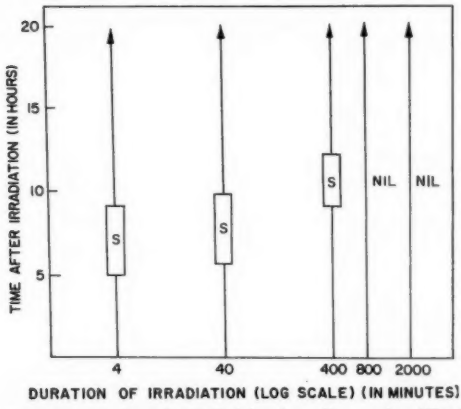

FIG. 2. Temporary suppression of cell division in pollen grains (*Tradescantia*) by a dose of 200 rads of X rays. The higher the dose rate (that is, the shorter the time of radiation), the more marked is the effect (S represents period of suppressed mitotic activity). At the lowest dose rates no effect is observed (after Alexander, "Atomic radiation and life", 1957, p. 52).

that the stage most sensitive to irradiation was the one at which the chromosomes divided. Experimental evidence to date indicates that the cell is most sensitive to damage from X irradiation during the latter part of the so-called resting stage and the very early part of prophase. The minimum radiosensitivity of chromosomes is at metaphase.

How are chromosomes broken? There are two extreme views to be considered: the theories of direct and indirect action. Most living systems contain a great deal of water and consequently when a cell or tissue is irradiated, most of the energy will initially be deposited in water. Changes produced by the reaction of these activated water molecules are referred to as indirect in contrast to direct action, which is a term applied to the changes which occur in the material which initially absorbed the radiant energy. The direct hit by an ionizing particle (electron, proton, alpha particle, etc.) might conceivably break a chromosomal thread, and lead to various anomalies in successive cell division. This might be called the physical explanation of chromosome breakage. An opposing view is to consider that ionizing radiations interfere with the potential of the cells to make chromosomal material. The chromosome break may then be considered a visible manifestation of an interruption of nucleoprotein synthesis. It may be that neither of these hypotheses is adequate and a new theory which incorporates features of both will have to be postulated.

The critical factor in chromosome breakage appears to be the number of ion pairs produced per unit of path length, and once a critical level has been reached, the probability of breaking a chromosome is high. Lea (1947) concluded that an ionizing particle can only break a chromosome if, in the track across a chromosome, 700 electron volts of energy are deposited. This is equivalent to about 20 ionizations (11). Densely ionizing radiations such as alpha particles are much more effective in breaking chromosomes than are

fast electrons. The average ionization density of electrons given off by ordinary X and gamma rays would not appear to possess enough energy to break a chromosome at all. However, an electron at the end of its track produces a sufficiently high ion density to break a chromosome. To summarize, Lea concluded that a chromosome is broken whenever an alpha particle, medium-energy proton, or an electron at the end of its track passes through it.

A blow to the above theory came with the discovery that the number of chromosomes broken by a given dose of X rays depended on the oxygen tension at the time of irradiation (12). In the complete absence of oxygen fewer breaks are found than with the same irradiation given in air. Breaks produced by densely ionizing radiations such as alpha particles depend to a less extent on the amount of oxygen present. On irradiation water is split up into free radicals $H\cdot$ and $OH\cdot$. These are highly reactive entities and $H\cdot$, to become stable, must lose an electron to give H^+ (hydrogen ion), and $OH\cdot$ must gain an electron to become OH^- (hydroxyl ion). The indirect action of irradiation can be explained in part by the reaction of these radicals on other organic substances in the cell in attaining once more the normal electron configuration. The radicals can also recombine with one another to re-form water, but other reactions are possible including the formation of H_2O_2, a highly reactive substance capable of damaging cellular structures.

Whether the above-mentioned radicals will react with dissolved substances or combine with one another depends largely on the ionizing ability of the radiant energy used. With alpha particles $OH\cdot$ radicals are produced in a high transient local concentration. No oxygen effect is observed and the important reaction is the combination of $OH\cdot$ radicals to yield H_2O_2. Only a small proportion of the $OH\cdot$ radicals are left to react with organic substances. In the case of beta, X, and gamma rays (sparsely ionizing radiations), a low local concentration of $OH\cdot$ radicals is produced and the reaction $H\cdot + OH\cdot$ with the production of water predominates. When oxygen is present, however, the radical $H\cdot O_2$ is formed. The amount of H_2O_2 produced also increases. Many free radicals are produced to react further with dissolved organic substances. It is possible with densely ionizing radiation that the direct action predominates, and with sparsely ionizing radiation that the indirect action is important in causing radiation damage. Most of the biological changes attributed to irradiation, from chromosome breaks to the dosage required to kill an intact organism, are decreased in the absence of oxygen during irradiation. The oxygen effect appears to be basic to radiobiology and to the understanding of effects of radiation on cells and tissues. Many of the biological effects of radiation can also be modified (in some degree) by other environmental factors such as temperature during and after irradiation, exposure to infrared radiation, protective agents, and others.

Radiation and Cell Chemistry

The current trend in research at the cellular level is aimed at the clarification of obscure and elusive injuries which cannot be seen under the microscope.

The study of the type of chemical reactions produced by radiation of various wave lengths and the processes by which the energy is converted into chemical change has contributed much to our understanding of the biological effects of ionizing radiations.

When irradiated in vivo, most proteins studied to date are very radioresistant. Even enzymes with SH groups like adenosine triphosphatase, hexokinase, and ribonuclease do not appear to be inactivated by doses which initiate profound biological damage in intact cells. Radiation effects on energy-yielding enzyme systems such as those of the Kreb's cycle do not appear to initiate the primary derangements of cellular function (13). In some cells the activity of these systems may even increase after irradiation. Incorporation of amino acids into proteins does not appear to be affected as a primary consequence of radiation injury. Experiments on the synthesis of nucleic acids, however, have given more encouraging results, and the inhibition of DNA syntheses has consistently been found in irradiated cells, whereas RNA syntheses or turnover is somewhat less affected and may even be increased (14).

In the last few years we have been using, as an experimental tool, the ciliated protozoan *Tetrahymena pyriformis*, single cells possessing mammalian-like nutritional requirements. These are grown on a chemically defined medium (15). Employing paper chromatographic techniques, starch gel electrophoresis, and other procedures, we have studied the possible effect of ionizing radiation on various metabolic processes. The transaminase system may be radiosensitive and has been studied particularly. Blocking this and other enzyme systems using radiation as a "molecular probe" we may increase our knowledge of cellular metabolism for it is entirely possible that other metabolic pathways may be used when established pathways are blocked.

Acknowledgment

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SOMATIC EFFECTS OF IONIZING RADIATION[1]

E. A. SELLERS

Abstract

Radiation is similar to other environmental changes in that the response or injury produced bears a relationship to the degree of stimulus. A time–dose relationship also exists. It differs from other environmental changes in that many more cells of the body remote from the skin surface are affected. The effects depend on absorbed tissue dose and on dose rate. These dosage factors, the varying sensitivity of the tissues, and differing rates of repair account for the phasic pattern of symptoms and tissue damage which follow radiation. Compared with other stressors (especially chemical) the dose–response curve after radiation is steep. The somatic effects of radiation are the sequelae of the specific molecular injury produced by ionization rather than the primary lesions themselves.

Altering the environment of an animal produces a series of responses characteristic of the specifically altered circumstance, yet with some common features. This is as true of exposure to ionizing radiation as it is of exposure to heat, to cold, to altitude, or to toxic substances. Regardless of the particular stressor (1), the degree and even the type of response (the injury) may be expected to vary with the extent of stimulus (or dose) and also to have a more or less specific time–dose relationship. Exposure to ionizing radiation differs from exposure to other environmental changes in that many more cells of the body, even those of visceral tissues remote from the skin surface, are subjected to the primary stimulus. The number and type of cells affected vary with the kind and quality of radiation, and of course with the proportion and the particular part of the body exposed; the injury varies accordingly. The energy and type influence the penetration and the amount of ionization which occurs along the track of radiation. The number of cells affected, the sites of cells in specific tissues, and the sites of ionization within cells will be determined by these factors. Unlike pharmacological agents, cell or tissue boundaries do not influence the dose distribution. While drugs often show pronounced molecular specificity in their interactions, ionizing radiation interacts with molecules along the track, irrespective of molecular structure. In considering the total dose received by the body or by an individual tissue, the factors already mentioned are important yet difficult to measure satisfactorily. It is clear that the energy and type of radiation, the methods of measurement, the size of experimental animals, and even the technique of exposing animals to radiation, the "geometrical factors", are of fundamental importance in experimental investigations. To illustrate the importance of tissue dose as contrasted to dose measured in air, the LD_{50} dose for rabbits was found in certain investigations to be 807 r or roentgens (measured in air) for 250-kev radiation and 1886 r for 80-kev radiation. The most likely explanation lies in the differing penetration of the radiation (2). The relative biological effectiveness is defined as the inverse ratio of the doses of two radiations required to

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produce a standard amount of a given biological effect. When radiation of 250-kev X ray is taken as unity, this varies from 0.7 with 20- to 30-Mev beta and gamma rays for many biological effects, to approximately 10 (slow and fast neutrons; cataract formation (3)). However, it should be emphasized that the term "relative biological effectiveness" depends on the criterion selected as the biological effect—lethality, spleen size, blood changes, etc.—and on the environmental and other factors which exist at the time. Cronkite and Bond discuss this at length (3).

Many of the confusing and differing results in the literature can be attributed to lack of information on or even to the disregard of such variables. Reference has been made in the previous papers of this symposium to the primary events which occur in cells and to secondary events which follow. In the intact animal some tissues appear to be radiosensitive in that signs of malfunction can be demonstrated easily in such tissues. Tissues in which rapid proliferation of cells normally takes place for the maintenance of proper function are most susceptible. Examples are the hemopoietic tissues, the mucosa of the gastrointestinal tract, the germinal epithelium of the testis, certain skin elements, and lenticular tissue. Injury or death of proliferative cells in time is necessarily followed by a deficiency of mature cells, e.g. leucocytes, erythrocytes, spermatocytes, or squamous cells. Debility of the whole organism will occur if the loss of mature cells is extensive enough to interfere with vital functions. In contrast to traumatic or thermal injuries or to the administration of most drugs, signs and symptoms of radiation injury except in very high dosage do not follow the stimulus immediately. After irradiation normal function of most tissues is continued until the store of mature critical cells is exhausted and only then do symptoms appear. Recovery of growing tissues will occur if death of the animal does not take place and if enough viable proliferative cells remain. The rate of recovery is related to the proliferative capacity of the cell types affected and the number of viable cells in the tissue. Sterility, epilation, and leucopenia are generally transitory in nature.

The recovery of somatic tissues from injury produced by radiation should be distinguished from the genetic effects produced by radiation. Here the effects are cumulative and no recovery occurs.

Because of the differing sensitivity of various tissues to radiation one might expect that the effects of radiation of the whole animal would follow a phasic pattern, that is, a pattern of injury and repair proceeding at different rates in numerous tissues, yet related with one another by the homeostatic mechanisms of the body. This is indeed the case. With very high dosage, e.g. 100,000 r, animals show widespread disorganization of all organ systems including those made up of relatively resistant tissues. A shock-like state ensues and death occurs within a matter of hours. This may be regarded as due to a failure of central co-ordinating centers—a "central nervous system death". Cellular destruction in the central nervous system can be demonstrated. With smaller doses (400–900 r) a delayed but still early death occurs with two peaks in the mortality curve. In rats one occurs between 3 and 5 days, the second at about 10 days. Deaths at the time of first peak are associated with ulceration and

hemorrhage of the gastrointestinal tract—"intestinal deaths". The later deaths are associated with anemia, hemorrhage, and extensive damage to lymphoid and myeloid tissues—"bone marrow deaths". At this time and later, the immune responses of the body are decreased and the animal is liable to infection. If the animal survives, the life span may be decreased and later effects such as graying of hair, cataracts, and an increased frequency of neoplasms are observed.

TABLE I

Penetrating ionizing radiation; acute radiation syndrome

	Determining tissue		
	Central nervous system	Intestine	Hemopoietic
Approximate dose	Over 4000	400	200
Latent period	1–3 hours	3–5 days	10–20 days
Predominant signs and symptoms	Lethargy Convulsions	Diarrhoea Fluid loss	Acellularity (bone marrow)
Time of death	2 days	8 days	30 days
Cause	Resp. arrest	Circ. collapse	Hemorrhage Infection

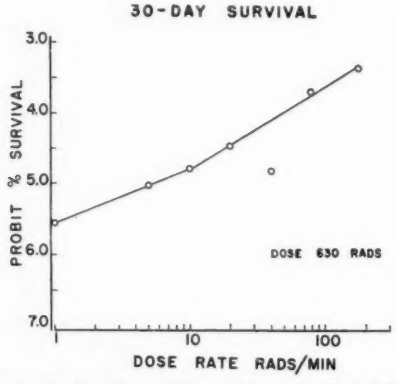

FIG. 1. The curve shows the effect of dose rate on survival (30 days). Groups of rats received a constant dose of 630 rads administered at different rates. The probit value of survival is higher with low dose rates than with high dose rates. The discontinuity in the curve suggests different causes of death (see text). (Figure kindly supplied by Dr. D. G. Baker.)

In the above discussion, no mention of dose rate has been made. The pattern of injury and recovery occurring together in many tissues, with different time relations, suggests that dose rate would be of importance. With rapidly administered radiation no chance for repair of a tissue is offered. With slowly administered radiation the processes of injury and repair proceed concurrently and those of recovery may equal or even exceed those of destruction. Depending on the dose and the dose rate, all variations may occur. Thus at one level of dosage, the symptoms and the cause of death, if it occurs, are pre-

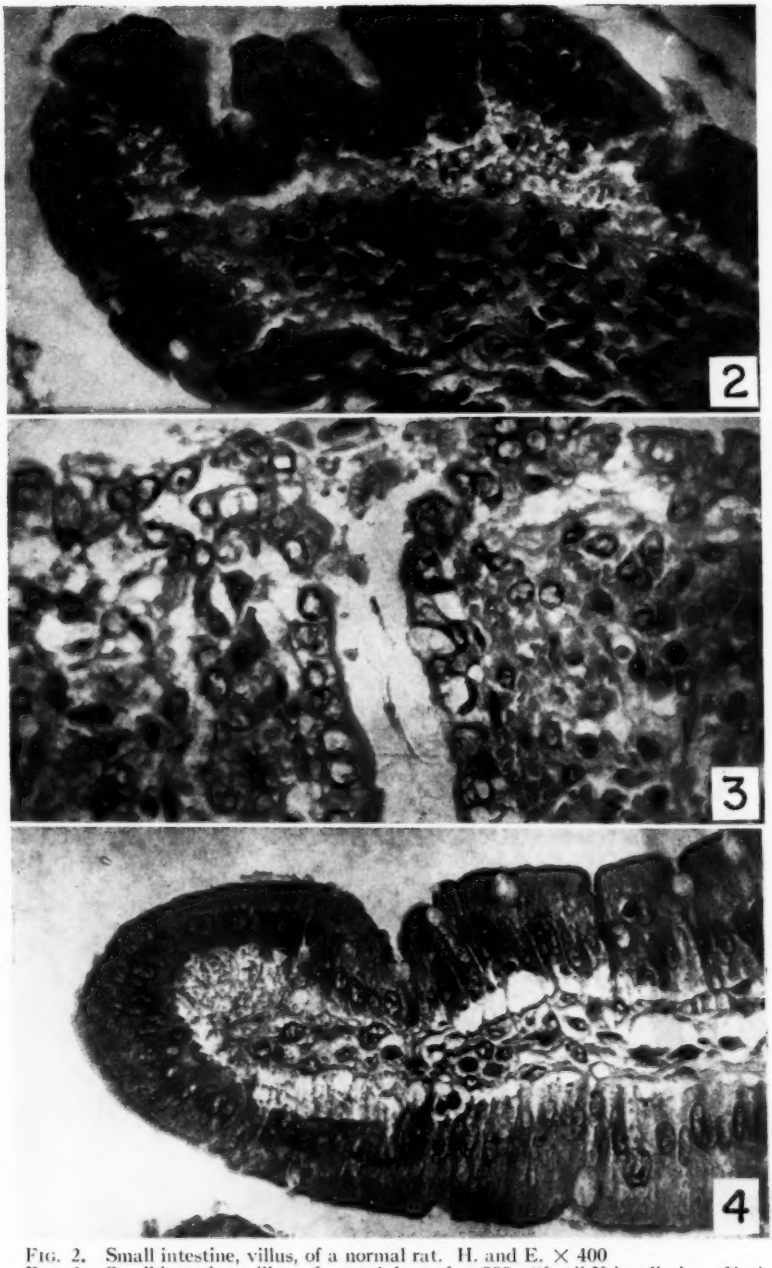

FIG. 2. Small intestine, villus, of a normal rat. H. and E. × 400

FIG. 3. Small intestine, villus, of a rat 4 days after 800 r "fast" X irradiation. Notice paucity of mucosal cells, change to cuboidal or flattened cells, irregularity of shape and staining of nuclei; congestion of submucosal capillaries, edema in submucosa, and disappearance of lymphoid cells. H. and E. × 400

FIG. 4. Small intestine, villus, of a rat 14 days after 550 r. The villus has regained its normal architecture; mucosal cells have been replaced with apparently normal cells. Lymphoid elements relatively scarce. H. and E. × 400

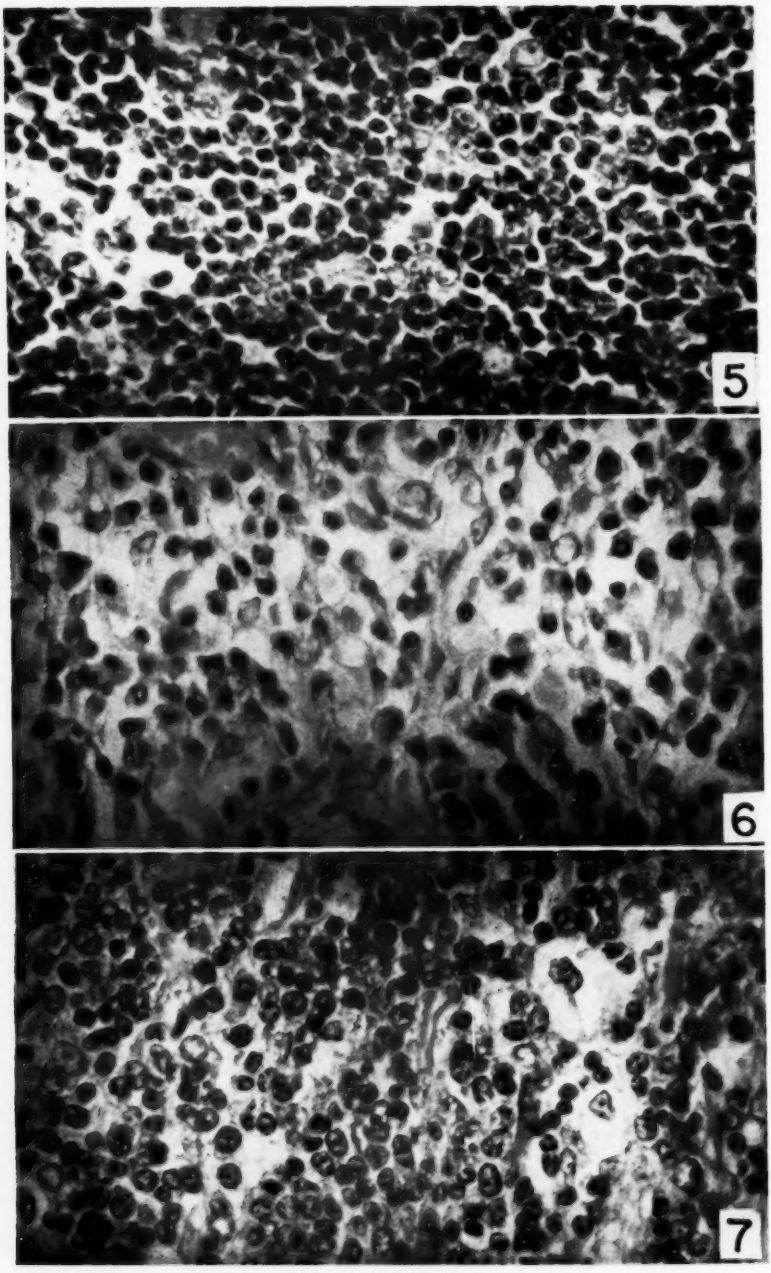

FIG. 5. Normal thymus, rat. H. and E. × 450

FIG. 6. Thymus, rat, 4 days after 800 r "fast" X irradiation. Note: edema, disappearance of lymphocytes, congestion, alteration in proportion of cell types. H. and E. × 450

FIG. 7. Thymus, rat, 14 days after 550 r. Note: repair has occurred, population of lymphocytes increased greatly. H. and E. × 450

dominantly gastrointestinal. At a lower level anemia and agranulocytosis, together with the loss of immune responses, lead to infection and death at a later date (Table I). With low continuous dosage only the late effects may be observed (Fig. 1). While accumulated small doses of radiation lead to a shortening of the life span and late effects (4), some evidence suggests that a type of adaptation may occur (5).

Twelve hundred roentgens administered within a few minutes would be expected to kill all mammals, while several times this dose given over 2 or 3 months would be expected to kill none. In practice, radiation delivered in 15 to 50 minutes is regarded as fast radiation and most descriptions of radiation injury refer to this type. Some of the changes observed after a midlethal dose of "fast" radiation are illustrated in Figs. 2, 3, 4, 5, 6, 7.

Perhaps some remarks on the common responses to injury mediated by the pituitary–adrenal system might be appropriate. Unlike other stressors radiation produces a primary effect on these glands themselves yet with moderately high dose rates signs of pituitary–adrenal stimulation occur. These are similar to those following other injuries yet are modified by the factors already discussed. Local death of tissues produced by radiation probably is responsible for much of the pituitary–adrenal response. Depending on dosage the effects elicited as a response to stress may loom large or be scarcely apparent. The changes elicited affect the outcome as they do with other forms of injury.

At first sight the interaction of radiation and organism would seem to present little similarity to drug–organism interaction, and some differences have already been pointed out. Some analogies exist. First, mode of administration can be compared with the characteristics of radiation, and both influence the tissues affected and the time course of events. Secondly, the dose rate in both cases influences the time course and type of effect. This has been pointed out for radiation. Pharmacologically, amphetamine administered rapidly causes death from its effects on the cardiovascular system, while prolonged administration results in death referable to the effects of the drug on the central nervous system (6). The effects of acute and chronic administration of other drugs like carbon tetrachloride or chloroform might also be mentioned.

It has been stressed that ionizing radiation is exceedingly toxic insofar as the lethal dose for mammals involves infinitesimal energy absorption and correspondingly a small number of primary chemical changes per cell. Pihl and Eldjam (7) have pointed out that certain pharmacological agents are equally toxic on a molar basis. Thus 0.5 mg per kg of nitrogen mustard gives rise to a concentration of 3×10^{-6} M assuming an even distribution. The LD_{50} of "fast" X-ray irradiation to the whole body is about 500 r, a dose which gives rise to 1.5×10^{-6} M ionizations and consequent chemical changes. Nevertheless, the dose – 30-day mortality curve is remarkably steep in the case of radiation. That is, the standard deviation of the LD_{50} dose is small. This is of particular interest because deaths occur extending a long period after the stimulus. In the case of chemical agents such a finding is usually associated with a less steep mortality curve. With radiation, if the LD_{50} dose is set at

unity, a standard deviation will follow in the range of 0.11 to 0.12 for several common species: the mouse, the rat, the hamster, the dog (8). These standard deviations are distinctly smaller than those given by Gaddum (9) for a number of pharmacological agents.

The somatic responses are governed by absorbed tissue dose and by dose rate. In these respects a field of radiation resembles any altered environment yet differs from most in that a relatively small change in these parameters produces a greater alteration in response. The physical signs of injury, and the symptoms they produce, vary not only in degree but even in obvious existence. From the point of view of producing acute somatic effects, with appropriate signs and symptoms, a series of "threshold" doses appears to exist. From the point of view of genetic or even late somatic effects, the "threshold" dose can be projected to zero or close to it. It will be clear that most of the somatic effects of ionizing radiation are the aftermath of the specific molecular injury produced by ionization. Their extent, their duration, and their importance are the sequelae of, rather than, the primary lesions themselves.

Acknowledgment

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GENETIC EFFECTS OF IONIZING RADIATION[1]

HOWARD B. NEWCOMBE

Abstract

Evidence relating to the probable genetic consequences of exposing large human populations to low levels of ionizing radiations is reviewed. There is little reason to doubt that the production of mutations varies linearly with dose even at very low doses, and that most of the mutations have undesirable effects. Estimates of the probable numbers of seriously affected individuals resulting from a given

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exposure can be derived from the present frequencies of hereditary diseases and the expected increases in mutation rate. The degree of uncertainty is probably about an order of magnitude in either direction. With doses similar to those received from nuclear weapon testing, estimates of this kind will seem small if expressed as a percentage of the total population but may look large if expressed in terms of the absolute numbers of seriously affected individuals in a world population of two and one-half billion people. Much unnecessary confusion has resulted from the seemingly conflicting statements which have been drawn from this single fact. The greatest single source of uncertainty stems from a basic ignorance of the genetics of human population and of the factors responsible for a present load of hereditary abnormalities and diseases affecting at sometime during their lives at least 4% of all infants born.

In spite of the impressive body of scientific literature describing the genetic effects of radiation in various organisms, and in spite of the existence of a number of recent authoritative reports dealing with the likely effects on man's own hereditary materials, there is still considerable popular misunderstanding concerning the consequences of exposing human germ plasm to ionizing radiation.

What most people want to know is whether the probable effects are trivial or important, and how sure we are. Among scientists there is a tendency to emphasize the second of these two questions, and perhaps the most useful function which I can perform in this symposium is to try to draw a careful distinction between what is well established and what is speculative, especially as relating to the genetic consequences of exposing large populations of people to low levels of radiation.

Dose–Effect Relationships

One basic premise which has not so far been seriously challenged is that the number of gene mutations resulting from irradiation varies in direct proportion to the dose. In other words, there is no threshold level of radiation below which the mutations will not be produced.

The evidence for this is reasonably good. The dose–effect relationships have been studied in a wide range of organisms over the last quarter of a century and intensive studies have been made in a number of these organisms. We have done work of this kind at Chalk River using mutations in microorganisms (1) and, as was later found by Russell (2) in mice, the response sometimes fell off at the higher doses (perhaps through selective death of the more sensitive cells) but showed no deviations from linearity at lower doses. Such studies, of course, become more difficult in the higher organisms than they are in the smaller and more rapidly breeding forms of life. In the fruitfly the curve has, by dint of considerable work, been pushed to within 25 roentgens of the origin (3, 4, 5). In the mouse, doses as low as 100 roentgens have been studied (6) but the confidence limits on the points are very much wider and the experiments are enormously laborious and expensive.

Rigorous evidence of a strictly linear response right down to zero dose would seem to be an almost unattainable ideal. However, this has been achieved in the bacteria using a change which is certainly not a typical gene mutation but which has much in common with the more familiar kinds of alterations of the genetic materials. This change involves the ability of a *prophage* which exists as a part of the bacterial gene string in certain so-called *lysogenic* bacteria to give

rise to infective virus particles. The system has been used with great refinement in studying the dose–effect relationships: the virus particles which have arisen from the rare spontaneous events can be removed with antisera; further spontaneous events can be prevented by keeping the bacteria in a non-metabolizing state; and the virus particles which arise as a result of small numbers of radiation-induced changes in large populations of bacteria can be counted with great accuracy following low doses of radiation which produce less than one ionization per bacterium. Under these conditions a strictly linear response at low doses down to the origin has been rigorously demonstrated (7, 8).

Thus one has a choice of evidence ranging from the limited observations on the vertebrates to the increasingly elegant experiments on organisms which are increasingly remote from man. However, the collective weight of the various lines of evidence in favor of a non-threshold response to the genetic effects of radiation is considerable, and we have no special reason to suppose that man is exceptional in this respect as compared with other living creatures.

This does not mean that the answers are all in, or that we are completely insured against the possibility of surprises in the future. At present, however, it would not be reasonable to assume that there is a threshold dose below which no mutations at all are produced.

It should be mentioned in passing that the results of one of the recent mouse experiments of Russell (6) have, in fact, been surprising in that they have shown a dose-rate effect, the yield of mutations being 2 to 3 times less when the dose was distributed over an extended period of time instead of being given as a single short exposure. This does not seem to affect the concept of linearity, however, since both curves took the form of a straight line with increasing dose in the lower dose range.

Russell's result indicates that the mutations which he is studying pass through an intermediate form which for a limited time is capable either of repair or of going forward to completion. The occurrence of other ionizations while the genes are in the indeterminate state influences this choice. There is also evidence of a somewhat similar phenomenon in *Drosophila*, and of a period of indeterminacy of about 30 minutes following the exposure (9). One practical consequence of this work might eventually be the setting of different permissible levels for human exposures depending upon whether the total dose had been received in a short or a long time.

The effort which has gone into demonstrating a linear relationship between radiation dose and gene mutation is quite exceptional. There has, however, been no comparable effort to extend the dose–effect relationships for somatic changes into the low-dose region (10, 11, 12). Hence, predictions of somatic hazards at low doses rest on an even more uncertain footing than do the predictions of genetic hazards and there is no certainty as to whether low doses have slight effects, or no effect at all, on the incidence of such conditions as leukemia and bone tumor.

Detrimental Versus Beneficial Mutations

Mutations when clearly recognizable almost always have detrimental effects, and by analogy it has sometimes been said that "one is unlikely to improve the performance of a clock by tinkering at random with its innards." The plant breeders are perhaps the experts on this since they use radiation as a means of obtaining agriculturally desirable mutations. A ratio of 1 "good" mutation for every 500 "bad" mutations has been reported by the Swedish workers using the "erectoides" mutation of barley (13). (Whether this mutation would be advantageous to barley in the wild state is open to argument.)

It does not necessarily follow that the ratio of "good" to "bad" is the same for mutations of a kind which produce slight quantitative differences and which would tend to pass unnoticed in the plant breeding experiments. However, James at Chalk River (14) has recently been studying yeast mutations of various degrees of severity which have quantitative effects upon the growth rate; he has found none with a clearly beneficial effect out of more than 50 which have been tested so far. Yeast growth rates lend themselves to very accurate quantitative measurement under controlled conditions, so that the conclusion that mutations are usually deleterious appears to apply both to mutations with the more striking effects and also to those with slight effects.

In apparent contrast to this, some evidence of a beneficial effect of induced mutations has been obtained from irradiated inbred fruitfly (*Drosophila*) populations (15). However, there is no actual conflict in the interpretation of these seemingly opposing results. The phenomenon of hybrid vigor has long been known. In the fly experiments, inbred lines were used and the introduction of mutant genes effectively led to the production of hybrid flies. Furthermore, the female fruitfly lays about 50 times as many eggs as are required to maintain the population at a constant level. For this reason *Drosophila* can afford to eliminate in each generation very large numbers of defective individuals, these being a small price to pay for even a slight population improvement. This situation has no analogy in human populations.

Effects on Humans

For practical reasons it has been necessary to form some opinion on whether the genetic consequences of exposing human populations to low levels of radiation are likely to be important or trivial. The levels which have had to be considered in this connection are: (*a*) the 3 roentgens per 30-year generation time which on this continent is the average accumulated exposure received by the reproductive tissues from the medical uses of X rays by the time an individual has reached the average age of parenthood (and which incidentally is approximately equal to the accumulated exposure to the natural background over the same period); (*b*) an exposure from fallout which with continued testing would be in the vicinity of 0.1 roentgen per 30 years; and (*c*) an additional amount which is received by the occupationally exposed but which at the present time is very small when averaged over the whole population.

Especially when considering occupational exposures, opinions as to the importance of the effects have had to be expressed in a usable form, i.e. in

the form of maximum permissible levels for industrial workers. Such levels must be reasonable both in relation to other more familiar risks and also in relation to certain anticipated benefits to society as a whole. Since neither the risks resulting from the exposures nor the benefits can be calculated with any degree of precision, it has sometimes been assumed that the natural level of 3 roentgens per generation probably does not have any major deleterious effect and that we cannot go too far wrong as long as the exposures to man-made radiation do not greatly exceed this level. The assumption is necessarily crude, and as sometimes stated it is even naïve, implying a faith in the gentleness of nature which may be quite misleading; but even a crude guiding principle is better than none at all.

It would, of course, be preferable to express the risks in terms of the probable numbers of seriously affected people.

Attempts in this latter direction are not always viewed with favor by scientists, some of whom fear that numerical estimates will carry with them the appearance of an accuracy which they do not really possess. The reasoning, however, is fairly straightforward and such estimates, far from being authoritarian in nature, have the special virtue of exposing both the reasoning and the assumptions, in a particularly naked form, to open scrutiny and criticism.

From the very laborious mouse experiments it has been estimated that about 30 roentgens would be needed to double the natural mutation rate of man in any one generation. The degree of uncertainty in applying these animal results to man is probably about threefold in either direction. The observations on humans who were exposed at Hiroshima and Nagasaki and their subsequent offspring (16, 17) were inherently too insensitive to detect any but a very large genetic effect. The absence of a detectable effect could therefore serve only one purpose, to limit the range of possibilities, and the conclusion has been drawn that the so-called "doubling dose" is very unlikely to be as low as the natural background of 3 roentgens and is probably not less than 10 roentgens. The favored figure might, however, have to be revised upward by two- or three-fold (i.e. from 30 up to 60 or 90 roentgens) as a result of Russell's recently observed dose-rate effect in mice.

Starting with the so-called "doubling dose", it is necessary to consider by how much the present amount of ill health would be increased if the mutation rate were to double. Fortunately there is good agreement on the prevalence of hereditary disease. About four out of every hundred liveborn infants will at some time in their lives be more or less severely affected by conditions which are largely determined by single gene differences. Lists of these diseases have been published elsewhere and need not be considered here (18). This 4% might conceivably be increased gradually over a number of generations to twice that value (i.e. to 8%) if the mutation rate were permanently doubled.

However, it is by no means certain whether most of these hereditary diseases are present because of repeated natural mutations. Alternatively many of the seemingly harmful genes might perhaps be maintained in the population because of some subtle benefit which they bestow, and which would result in an increased fertility or survival of the individuals who carry the genes but who are not themselves ill. This possibility seems unlikely in the more severe

and highly penetrant dominant conditions such as certain forms of blindness, deaf-mutism, and dwarfism. In these, mutation is probably the culprit, but direct evidence is unfortunately extremely limited. In fact, we are not really sure whether the bulk of the natural "load" of hereditary defects is present because of repeated mutations or because of some subtleties of natural selection.

Until we know more about the genetics of human populations, and in particular about the respective roles played by natural mutation on the one hand and by fertility differentials on the other in maintaining the present load of hereditary diseases, it will be difficult to specify how much effect to expect from a given increase in the mutation rate. This absence of an adequate body of basic information relating to the genetics of human populations probably represents the most serious source of uncertainty in predicting the genetic consequences of exposing human populations to radiation. For this reason a considerable part of my own research activity in the last few years has centered on the development of methods by which the appropriate human population studies might be carried out on an adequate scale at a reasonable cost.

In the meantime, however, there are fairly strong reasons for believing that at least a quarter of the diseases represented in the 4% (the more severe and clearly dominant conditions) would double in incidence with a doubling of the mutation rate, and that all of this number might conceivably do so. For simplicity of calculation one can use the figure of 2% with an uncertainty of at least twofold in either direction.

This reasoning permits one to calculate the likely numbers of seriously affected individuals within rather wide limits. There are some oversimplifications which I will not take the time to describe in detail (namely the inherent assumptions (*a*) that the world population is static, when in fact it is doubling about every 50 years; (*b*) that the present load of hereditary defects represents an equilibrium level, when it is quite possible that we are moving towards a new equilibrium as a result of medical and social changes; and (*c*) that a neglect of the more "quantitative" hereditary characters such as longevity, intelligence, and "fitness" will not lead to any gross underestimation of the hazard).

I should like to mention briefly a paradox which has resulted from such calculations. The hazard to large populations from exposure to low levels of radiation will seem extremely small if expressed percentagewise, but if expressed in terms of the numbers of affected individuals per generation in a world population of two and one-half billion, or in a future world population of five billion or more, it may well seem large indeed. To illustrate this point let us assume for the moment, somewhat pessimistically, that a continued exposure of the world population to an additional 1/10th roentgen per generation would result in the future in an additional one million seriously affected people per generation. This falls within the wide range of possibilities indicated by the preceding reasoning. And yet, even such a large number is only 1/25th of 1% of a world population of 2.5×10^9 (and is, from the standpoint of the individual, considerably less than the 30-year risk of a fatal automobile accident in Canada). Clearly, it is possible to say that the hazard to future individuals from such a small rise in the radiation level is very small indeed, and yet it can equally

well be said that a very large number of individuals may be seriously affected. Such seemingly conflicting statements have served to perpetuate a confusion which is largely semantic in origin, but which in the public mind has implied a fundamental lack of agreement. Actually there is very little real debate about the nature and extent of the areas of genuine ignorance, and it is time we got away from this unnecessary and unscientific kind of controversy and concentrated on getting the information which we most need.

In this particular example I have chosen an extremely low dose which is perhaps comparable to that expected from continued weapon testing. The much higher exposure of 3 roentgens per individual per generation which on this continent is received as a result of the medical uses of X rays will of course have a correspondingly greater genetic effect. However, in such exposures the medical needs of the individual must be weighed against the possibility of damage. In spite of the very wide limits associated with the estimates of the hazard obtained by calculations of this kind, such estimates probably constitute the best available objective aid for the purpose of balancing the benefits from various procedures against the possible detriment. Certainly the results of such calculations are not alarmist, but they have raised the question of whether the same diagnostic results can in some cases be achieved with lower doses or with fewer exposures.

Finally whether there is peace or war we will in the future be making increasing use of very potent sources of radiation, probably for the rest of our civilized existence. If sound judgment is to be exercised concerning the radiation levels to which human populations can justifiably be exposed, there will have to be a progressive increase in our understanding of the consequences. It is perhaps not generally recognized that some of the most pressing problems can only be solved by a particularly laborious and difficult kind of long-term research, and yet this is true for the very simple reason that these problems relate to low dose levels and to delayed and relatively rare effects occurring in large populations. Therefore, any experiments with laboratory animals or statistical studies of human populations will of necessity involve large numbers of individuals or families who may have to be studied over their whole lifetime, or even for many generations. Our understanding of the effects on human populations will undoubtedly depend much upon the rate at which we undertake these more difficult and less attractive lines of research.

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QUESTIONS AND DISCUSSION

Dr. D. G. Baker, University of Toronto

To understand the very complex interactions which exist when biological material is exposed to ionizing radiation, we must pursue our investigations at all levels of biological organization. Through the collaboration which I have enjoyed with Dr. Clarke, I have a special interest in this type of investigation. There are many more questions than answers. For example, using the two test systems he mentioned, a very pronounced dose-rate effect has been observed. One wonders if there exists a similar phenomenon for tumor cells. Perhaps, for a given variety of tumor cell, there might be a dose rate which is optimal to produce injury. One might, by selecting the dose rate, be able to damage tumor cells while subjecting normal tissue to a minimal amount of radiation injury. Both Dr. Whitmore and Dr. Clarke indicated that relatively small amounts of radiant energy need be absorbed by the cell to produce modifications of function. I would ask Dr. Clarke if he feels that these modifications of cell function after exposure are primarily due to the action on nuclear material (nucleoproteins), DNA, or DNA synthesis, or are there some other mechanisms equally important?

Dr. G. M. Clarke, University of Toronto

There has been a great deal of work done on DNA synthesis. One of the observable effects is on DNA and RNA synthesis and on respiration of the cell. Daniel Mazia and others have transferred the nucleus from an irradiated amoeba to a non-irradiated amoeba and vice versa. Cells with an irradiated nucleus usually survive 3 days, whereas the amoeba with the irradiated cytoplasm could live for as long as 3 weeks. The greater resistance of the cytoplasm to radiation injury is probably related to the fact that it contains many multiples of important structures; the nucleus in contrast rarely possesses more than two sets of chromosomes. Mazia mentions this work (Section 4 of the National Academy of Sciences, N.R.C. Report No. 18, 1956). One thing should be commented upon. People probably wonder why we are working with buckwheat seeds. We hope to obtain seed and plants with variations in chromosome number. We already have a 16- and 32- chromosome species. We are interested in the fact that with increasing chromosome number there is a decreasing radiosensitivity. Dose and dose-rate studies, coupled with studies on the mitotic cycle, may one of these days give information which may be of benefit to people engaged in radiation therapy.

Dr. George Weber, Montreal Cancer Institute, Research Laboratories, and University of Montreal

I appreciate Dr. Sellers' invitation to comment and, since he sent me his paper before the symposium, I prepared my remarks to illustrate on the biochemical level some of the problems which he discussed.

In our laboratories during the past 6 years the effect of irradiation on mammalian systems was investigated by following the biochemical sequence of events in normal and hypophysectomized animals subjected to 600 r total body X irradiation. On the basis of mortality curves, animals were killed for biochemical studies at 1, 3, 7, and 8 days post-irradiation.

Normal rats subjected to irradiation exhibited a characteristic metabolic response, manifested by marked changes in enzymatic activities and metabolite levels. Blood sugar, liver glycogen, and nitrogen levels steadily increased to a peak value at 3 days, and returned to normal range by the 8th-day post-irradiation. The presence of increased gluconeogenesis and glycogenesis was supported by the fact that hepatic phosphohexoseisomerase and phosphoglucomutase activities paralleled the alterations in liver glycogen concentration (Radiation Research, **7**, 459 (1957)). Hepatic glucose-6-phosphatase activity was not affected by irradiation, whereas 6-phosphoglyconate dehydrogenase rose to twice the pre-irradiation activity and remained at this high value throughout the post-irradiation period (Weber and Cantero).

In contrast, in *hypophysectomized* rats irradiation caused no change in blood sugar, hepatic glycogen, and carbohydrate enzymatic activities. Thus hypophysectomy abolished the ability of the organism to exhibit the characteristic metabolic response to irradiation.

Liver carbohydrate enzymes in vitro were not affected by irradiation doses up to 30,000 r, indicating that the described in vivo alterations were not caused by direct effects of irradiation but were mediated through hormonal alterations.

Studies on the over-all behavior of metabolic pathways in irradiated animals showed that liver glycolysis closely paralleled the changes in hepatic phosphohexoseisomerase activity. Liver respiratory activity was not affected by irradiation (Rev. can. biol. **18**, September 1959).

Our studies suggest the necessity of careful evaluation of biochemical alterations occurring after exposure to irradiation to separate possible direct effects of irradiation from those mediated through alterations in the hormonal and nutritional status of the organism.

These results are in line with the principles emphasized in the paper of Dr. Sellers.

Dr. Stanley G. Skoryna, McGill University

The discussion of biological effects of ionizing radiations would be incomplete without considering their carcinogenic action. Within the time limit allotted, I wish to summarize the important features of these effects. It is generally recognized that the carcinogenic effects of ionizing radiations can take place either in a direct or an indirect manner; the best examples of these two modes of tumor development are probably the induction of bone cancer by radioactive strontium and leukemia, following total body irradiation, respectively. In both instances, similarly to other carcinogenic agents, the electron potential, duration of exposure, type, dose level, and a number of modifying factors are of importance. However, I wish to emphasize here that ionizing radiations belong to the group of most potent carcinogens, although they play a relatively minor role in the etiology of human neoplasia. We have recently drawn attention to the non-specificity of inducing factors of common tumors in man; Salamon and Roe have suggested the division of carcinogenic agents into complete and incomplete. Although the line of division is not clear in many cases, ionizing radiations can be definitely counted among the complete carcinogens, as they require relatively few additional factors, and natural resistance can be frequently overcome by proper selection of time–dose relationships. This statement refers only to high-dosage irradiation. It does not imply that low doses of the same type of ionizing radiation would act in a similar manner.

The experience of our group at McGill University with induction of bone tumors in rats by radioactive strontium administration confirms such course of events. However, the maximal permissible concentration which lies below the tumor dose is difficult to determine within our present knowledge: the ionizing radiation might not be evenly distributed in the body; factors affecting cumulation are frequently unknown; and finally the co-carcinogenic action in development of spontaneous tumors has to be considered.

The increasing use of ionizing radiation in industry and science and the problems of fallout make it imperative to advance our limited knowledge of carcinogenic action of ionizing radiations. Dr. Taylor of the National Cancer Institute has recently stated that radiation is the problem of our time. To this I wish to add that carcinogenic effect represents one of the principal components of this problem.

QUESTION.—How does the discovery by Howard-Flanders that nitric oxide imitates the oxygen radiosensitivity effects fit in with the simple kinetics of radicals which you presented?

Dr. J. W. Hunt, Princess Margaret Hospital, Toronto

The recent work by Flanders and by Gray and his associates has shown that nitric oxide has much the same effect on radiation sensitivity as does oxygen, when applied to systems where the nitric oxide is non-toxic. The fact that nitric oxide does exhibit this effect throws considerable doubt on the mechanism of the oxygen effect. However, both oxygen and nitric oxide are scavengers of free radicals and so perhaps nitric oxide could react in much the same way as Dr. Whitmore has depicted for oxygen. On the other hand, both oxygen and nitric oxide are paramagnetic substances and it may be that it is their paramagnetic properties which account for the radiation sensitization.

Dr. F. Bohatirchuk, University of Ottawa, commented on the absence of a threshold dose of radiation insofar as genetic effects are concerned but that differing effects occurred with different doses and types of radiation. This fact has been recognized in radiation therapy. He noted that we live in an environment of radiation and the cumulative effects of low doses are not well known. He stated that in considering the increased rate of mutations produced by low doses of radiation, all factors influencing rate of mutations should be considered.

PROGRAM OF THE FIRST INTERNATIONAL CONGRESS OF ENDOCRINOLOGY

The program of the First International Congress of Endocrinology, which will be held in Copenhagen, Denmark, July 18–23, 1960, will consist of 10 symposia and a round table discussion, along with sessions of communicated papers. The speakers at the symposia and the round table discussion have been invited by the Subcommittee on Program of the Congress. In symposia Nos. 1–8 and in No. 10, the Subcommittee has also invited a discusser for each principal paper. Symposium No. 9 will consist of six principal papers and no discussers. The round table discussion will consist of eight sections, each with a principal speaker and three secondary speakers. This program of invited speakers is as follows:

1. *Central nervous system regulation of anterior pituitary secretion.* July 19, 9–12 a.m.

J. Benoit, France: Système nerveux central et contrôle de la fonction gonadotrope préhypophysaire. Discusser: S. Katsuki, Japan.

G. Harris, England: Central nervous control of gonadotrophic and thyrotrophic secretion. Discusser: L. Desclin, Belgium.

H. D. Purves, New Zealand: Hypothalamic control of thyrotrophin and prolactin secretion. Discusser: J. J. van der Werff ten Bosch, Netherlands.

G. Sayers, U.S.A.: Mediation of hypothalamic control over corticotropin release. Discusser: R. J. Guillemin, U.S.A.

2. *Posterior pituitary hormones.* July 19, 1–3 p.m.

H. Croxatto, Chile: Natural and synthetic related substances having neurohypophyseal hormone-like activities. Discusser: R. A. Boissonas, Switzerland.

R. Caldeyro-Barcia, Uruguay: Oxytocin in pregnancy and labor. Discusser: F. Stutinski, France.

H. Heller, England: Antidiuretic hormones of the neurohypophysis. Discusser: L. Martini, Italy.

3. *Parathyroid hormones and calcium metabolism.* July 19, 3.30–5.30 p.m.

H. Rasmussen, U.S.A.: The nature and properties of the parathyroid hormone. Discusser: P. Munson, U.S.A.

W. F. Neuman, U.S.A.: Experimental biology of the parathyroid secretions. Discusser: P. J. Gaillard, Netherlands.

T. R. Fraser, England: Clinical tests of parathyroid abnormality. Discusser: F. C. Bartter, U.S.A.

4. *Anterior pituitary hormones.* July 20, 9–12 a.m.

A. B. Lerner, U.S.A.: The mechanism of action of the melanocyte stimulating hormone. Discusser: K. Hofmann, U.S.A.

C. H. Li, U.S.A.: Biochemical endocrinology of pituitary growth hormone. Discusser: M. S. Raben, U.S.A.

R. Luft, Sweden: Studies on the metabolic action of human growth hormone. Discusser: J. Gillman, South Africa.

J. A. Loraine, Scotland: Gonadotrophin assays as an index of pituitary function in man. Discusser: C. A. Gemzell, Sweden.

5. *Adrenocortical syndromes.* July 20, 1–3 p.m.

D. J. Ingle, U.S.A.: The relationship of the adrenal glands to diseases of adaptation. Discusser: T. Symington, Scotland.

T. F. Gallagher, U.S.A.: Studies of steroid metabolism in man. Discusser: T. F. G. Prunty, England.

L. Wilkins, U.S.A.: The treatment of virilizing adrenal hyperplasia. Discusser: J. Decourt, France.

6. *Aldosterone.* July 20, 3.30–5.30 p.m.

G. Farrell, U.S.A.: Influence of the mesencephalon and epiphysis cerebri on adrenal function. Discusser: A. F. Muller, Switzerland.

F. Gross, Switzerland: Regulation and mechanism of sodium transport by aldosterone and related corticoids. Discusser: H. P. Wolff, Germany.

J. W. Conn, U.S.A.: The evolution of primary aldosteronism from 1954 to 1960. Clinical manifestations, pathology, and the results of adrenal surgery. Discusser: F. Tronchetti, Italy.

7. *Aspects of reproduction.* July 22, 9–12 a.m.

A. Jost, France: The effect of sex-related steroids on embryonic sex differentiation and growth. Discusser: C. W. Emmens, Australia.

H. Nowakowski, Germany: Congenital disorders of the testis. Discusser: J. Charvat, Czechoslovakia.

E. Diczfalusy, Sweden: Endocrine functions of the human placenta. Discusser: J. Zander, Germany.

G. Pincus, U.S.A.: Fertility control by endocrine agents. Discusser: W. O. Nelson, U.S.A.

8. *Steroid pharmacology.* July 22, 1–3 p.m.

A. Zaffaroni, Mexico: The effect of alkyl- and electronegative group substitution on steroidal hormonal activity. Discusser: M. Tausk, Netherlands.

R. Hertz, U.S.A.: The pharmacological alteration of the biosynthesis and the peripheral effects of steroid hormones. Discusser: T. Nakao, Japan.

J. Ferin, Belgium: Comparative assays of new sex steroids in the human. Discusser: G. Sala, Italy.

9. *Hormone action at the cellular level.* July 22, 3.30–5.30 p.m.

R. Michel, France: Pénétration et utilisation cellulaire des hormones thyroïdiennes.

P. Talalay, U.S.A.: The participation of steroid hormones in enzymatic reactions.

O. Hechter, U.S.A.: Cell permeability and hormone action.

E. W. Sutherland, Jr., U.S.A.: Formation of cyclic 3,5-adenylic acid and its relation to the action of several hormones.

P. J. Randle, England: Interactions between metabolism and sugar transport in muscle as a site of insulin action.

N.-A. Hillarp, Sweden: Storage and release of catechol amines.

10. *Topics in comparative endocrinology.* July 23, 9–12 a.m.

O. Mühlbock, Netherlands: Hormonal factors in tumorigenesis and tumor growth. Discusser: O. Pearson, U.S.A.

C. M. Williams, U.S.A.: Present understanding of the juvenile hormone of insects. Discusser: P. Karlson, Germany.

L. Gallien, France: Etude comparée des activités des hormones stéroïdes dans la différenciation sexuelle des amphibiens. Problèmes de spécificité et effets paradoxaux. Discusser: F. Pautsch, Poland.

N. L. Gerbilskii, U.S.S.R.: Endocrinology of fishes. Discusser: E. C. Amoroso. England.

Round Table Discussion: The validity of hormone assays applicable to clinical medicine. July 23, afternoon.

1. Corticosteroids. R. H. Silber, U.S.A.; secondary speakers: J. K. Norymberski, England; R. I. Peterson, U.S.A.; R. V. Brooks, England.

2. Androgens. R. I. Dorfman, U.S.A.; secondary speakers: C. J. Migeon, U.S.A.; L. O. Plantin, Sweden; A. E. Kellie, England.

3. Aldosterone. J. F. Tait, U.S.A.; secondary speakers: R. Neher, Switzerland; E. H. Venning, Canada; J. A. Luetscher, Jr., U.S.A.

4. Progesterone, pregnanediol, and pregnanetriol. R. Borth, Switzerland; secondary speakers: A. Klopper, Scotland; R. V. Short, England; A. M. Bongiovanni, U.S.A.

5. Gonadotrophins. A. Albert, U.S.A.; secondary speakers: C. Hamburger, Denmark; B. Lunenfeld, Israel; R. Henry, France.

6. Estrogens. J. B. Brown, Scotland; secondary speakers: J. R. K. Preedy, U.S.A.; M. F. Jayle, France; G. Ittrich, Germany.

7. Thyroid hormones and thyrotrophin. A. Querido, Netherlands; secondary speakers: J. B. Stanbury, U.S.A.; P. M. Bottari, Belgium; J. E. Rall, U.S.A.

8. Catechol amines. U. S. von Euler, Sweden; secondary speakers: F. Elmadjian, U.S.A.; M. D. Armstrong, U.S.A.; H. Weil-Malherbe, U.S.A.